Central dopamine and oxytocin interactions during penile erection

by

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PhD by research

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This thesis has been composed by the candidate, Tracey Baskerville and the work is that of my own and the work has not been submitted for any other degree or professional qualification except as specified.

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Publications

Published papers

- Baskerville TA and Douglas AJ. 2007. *Interactions between dopamine and oxytocin in the control of sexual behaviour,* Progress in Brain Research – *in press*, 2008.

Published abstracts

- Baskerville TA, Douglas AJ. Jun 2006. *Increased Fos Expression in Paraventricular Nucleus Oxytocin Neurons Following D2 but not D4 Receptor Activation.* International Conference for Neuroendocrinology (combined with Society for Behavioural Neuroendocrinology), Pittsburgh, USA.
- Baskerville TA, Douglas AJ. Nov 2006. *Quinelorane Activates Oxytocin Neurones in the Hypothalamus During Penile Erection.* Society for Endocrinology, London, UK.
- Baskerville TA, Douglas AJ, Allard J. Mar 2007. *Paraventriculospinal pathways modulate apomorphine-induced penile erection.* University of Edinburgh Neuroscience Day, Edinburgh, UK.
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- Baskerville TA, Douglas AJ, Allard J. Jul 2007. *Paraventriculospinal pathways modulate apomorphine-induced penile erection.* Life Sciences, Glasgow, UK.
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- Baskerville TA, Addison M, Douglas AJ. Nov 2007. *The differential effects of dopamine D2-like receptor antagonists on intromission-induced Fos expression in oxytocin neurons in hypothalamic nuclei of the male rat.* Neuroscience 2007, San Diego, California, USA.

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Abstract

Central dopamine and oxytocin interactions during penile erection

Both dopamine and oxytocin have established roles in the central regulation of male sexual behaviour in rats and are clearly pro-erectile when exogenously applied. Penile erection induced by dopamine or oxytocin can be blocked by pre-treatment with an oxytocin and dopamine receptor antagonist, respectively (Martino et al 2005; Melis et al, 1997). So it is believed that an interaction exists between these two neural circuitries during the stimulation of penile erection. However, where such neural crosstalk occurs and by what cellular mechanisms remains unknown. The medial preoptic nucleus (MPN), supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus are three brain nuclei believed to have an important role in masculine sexual behaviour. To investigate a dopamine-oxytocin link in the central regulation of erectile function, this study was comprised of three parts to examine (1) the behavioural effects of dopamine agonists and antagonists on penile erection-induced neuronal activation within the three key hypothalamic nuclei (2) the expression of D2, D3 and D4 receptor expression by hypothalamic oxyocin neurons and (3) the effect of spinal oxytocin receptor blockade on dopamine agonist –induced penile erection. The data show that whilst D2 and D3 but not D4 receptor stimulation during pharmacological-induced penile erection appears to involve in part the activation of parvocellular paraventricular oxytocin neurons, magnocellular oxytocin cells in the hypothalamic nuclei remain unaffected. Conversely, blockade of central D4 but not D2 or D3 dopamine receptors during physiologically-induced penile erection, attenuated the activation of magnocellular supraoptic oxytocin cells and was without effect on paraventricular oxytocin cells. Additionally, antagonism of central D3 receptors significantly decreased neuronal activation in all three regions of interest after intromission, however, these neurons were not oxytocinergic. Thus, dopaminergic stimulation (and/or the behavioural context) of penile erection may involve differential activation of central oxytocin neurons. In addition, D2, D3 and D4 receptors were expressed by oxytocin cells in the MPN, SON and PVN with D4 receptors predominantly expressed on magnocellular oxytocin neurons. Finally, blockade of oxytocin action in the lumbosacral cord (a potential release site for oxytocin), markedly impaired erectile function after dopaminergic stimulation. Taken together, the data suggest that dopamine elicits penile erection via

the stimulation of D2, D3 and/or D4 receptors (perhaps located on oxytocin neurons) by differential activation of non-oxytocinergic and/or oxytocinergic pathways in specific hypothalamic nuclei. Additionally, such dopamine-mediated penile erection may involve a modulatory role of spinal oxtyocin.

Chapter 1

General Introduction

Central control of penile erection

Chapter 1

General Introduction

1.1 Introduction

Male sexual behaviour is a well characterised and relatively highly conserved behaviour in most mammals. Male sexual behaviour is a complex series of behavioural events that culminates in ejaculation. It involves the integration of sensory, olfactory and social cues which subsequently stimulate central and peripheral processes to elicit penile erection and co-ordinate skeletal and motor output that facilitate copulation. Sexual behaviour is comprised of a motivational phase (sexual arousal and mounting) and a consummatory phase (penile erection, intromissions and ejaculation). The mating pattern consists of a brief number of mounts followed by a series of intromissions (approximately 8-15) until ejaculation occurs. The rat enters a brief sexual refractory period (approximately 6-10 min) before engaging in further sexual contact (Beyer et al, 1981; Paredes and Agmo, 2004 for review). The brain loci involved, neurochemical correlates and the neural circuitry underlying each sexual event (namely, sexual motivation, mounting, penile erection, ejaculation, sexual reward and sexual satiety) have yet to be fully elucidated. However, much progress has been made in recent times with the use of more highly refined lesioning, tract-tracing and immunocytochemical techniques as well as the commercial availability of more pharmacological tools with greater selectively, to identify specific brain nuclei and neural substrates that may mediate components of masculine sexual behaviour.

1.2 Penile erection

Penile erection is one particular component of male sexual function that has received much attention due to reports of erectile dysfunction in those patients suffering from a range of diseases including diabetes, andrological and psychotic disorders (Basu and Ryder, 2004; Glander et al, 2007; Papatsoris et al, 2006). Although psychogenic factors may have a contributory role in erectile dysfunction, organic processes including vascular, hormonal and neurobiological are the main leading causes of the pathophysiology of erectile dysfunction (Kaminetsky, 2008). Penile erection is a complex reflex

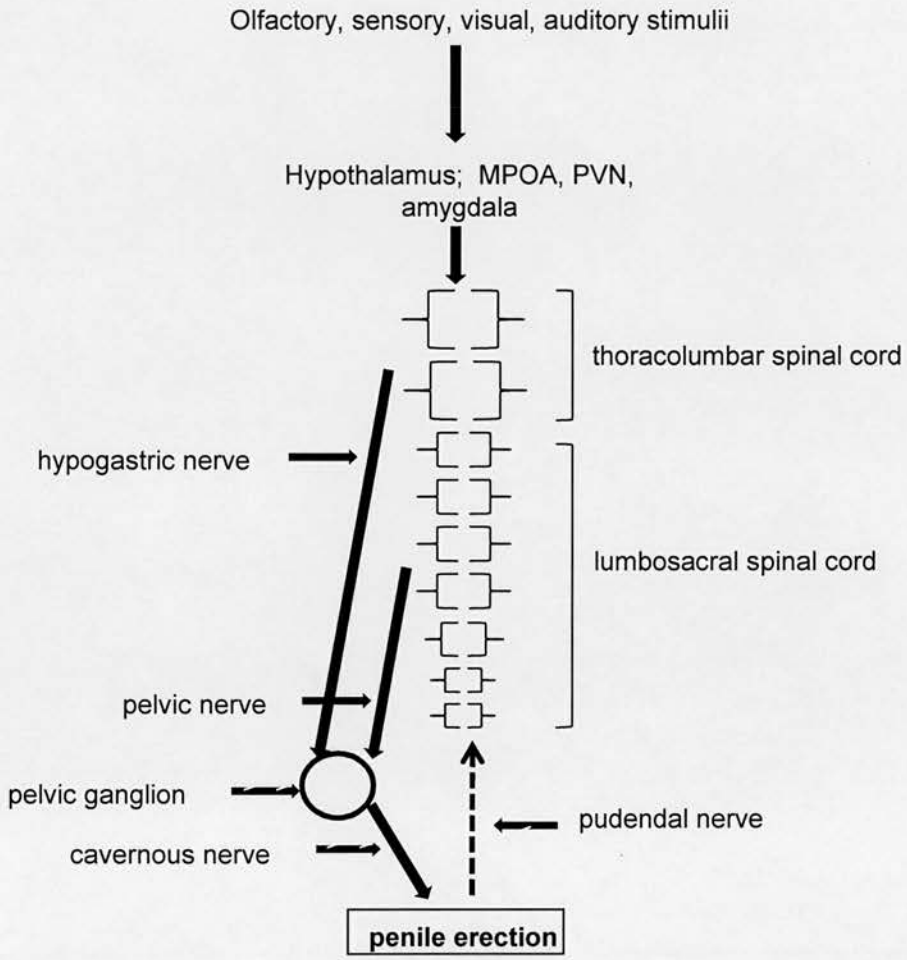
response influenced by neural, steroidal, hormonal and vascular inputs (Giuliano and Rampin, 2000; Heaton, 2000; Hull et al, 1999; Ralph, 2005); the neurochemical and anatomical properties of which have yet to be fully established. Achievement of penile erection relies upon increased blood flow to the penis (via arterial dilatation), engorgement of the penis with blood and penile rigidity (due to relaxation of the corpus cavernosum and corpus spongiosum) and contraction of the perineal striated muscles to enhance penile rigidity. Autonomic pathways originating in the spinal cord serve as the main anti-erectile (sympathetic) and pro-erectile (parasympathetic) pathways controlling penile erection. The intraspinal networks responsible for preventing and eliciting penile erection are the thoracolumbar sympathetic and sacral parasympathetic nuclei, respectively, (Giuliano and Rampin, 2000 for review). Excitatory signals are transmitted to the penis via the cavernous nerve which receives input from the pelvic nerve. Because of the close anatomical arrangement between spinal networks and the penis and studies showing spinal cord injured rats and humans (thus removing all supraspinal contributions) are capable of eliciting penile erection (Chapelle et al, 1980; Ishizuka et al, 2002; Vargas et al, 2004); the spinal cord is emerging as the key structure controlling penile erection which integrates converging commands from peripheral and supraspinal influences.

From the spinal cord, sympathetic and parasympathetic fibres running in the cavernosal nerves provide the neuronal efferents to the penis. The cavernosal nerves reside in the major pelvic ganglia, which are in turn innervated by lumbosacral-derived pelvic nerves and thoracolumbar-derived hypogastric nerves. Additionally, the sensory afferents of peripheral origin are relayed to the lumbosacral spinal cord via the pudendal nerves and impinge on the dorsal penile nerve (Figure 1.1).

1.3 Intromission

The term intromission is used in rodent sexual behaviour studies and serves as a physiological marker of penile erection. Intromission is often defined as *in-copula* penile erection and is a highly stereotyped behaviour. In rats, achievement of intromission occurs when a male is placed with a receptive female rat. Male rats initially display mounting behaviour (when the male positions himself directly behind the female and place's his front paws on her back without displaying pelvic thrusting) during which, the genital region receives somatosensory stimulation. Such genitosensory feedback

Figure 1.1: Neuroanatomy of penile erection



Neuronal pathways regulating penile erection. Sexually-associated stimulii reach the penile erection centres in the brain, including the hypothalamus. Neural signals are transmitted to the spinal cord where anti-erectile sympathetic fibres run in the hypogastric nerve toward the pelvic ganglion. Likewise, pro-erectile parasympathetic pathways present in the pelivc nerve also impinge on the pelvic ganglion. For both pathways, the cavernous nerve serves as the final common pathway to the penis. Sensory information from the penis is relayed to the spinal cord via the pudendal nerve.

(Adapted from Argiolas and Melis, 1995)

accompanied by sexually-stimulating olfactory cues act to facilitate intromission in the male rat. During intromission, *in-copula* stimulation of the prepuce seems to have a role in the activation of copulatory thrusting patterns (Contreras and Agmo, 1993). Once the thrusting threshold has been reached, the combined peripheral inputs from the penis, prepuce and perineal striated muscles to the brain may result in the activation of as yet unknown central pathways culminating in penile withdrawal and dismounting. Intromitting parameters such as latency and frequency are often recorded during copulation and they can be regarded as indicators of penile erection associated with sexual arousal and erectile efficiency, respectively.

1.4 Ejaculation

Ejaculation is essentially a physiological process and can be difficult to define behaviourally, even to the trained observer. It is a spinal reflex and is comprised of two phases: seminal emission and expulsion. Seminal emission involves secretion of seminal fluids into the urethra and contraction of the vas deferens to propel the sperm through this vessel. Expulsion of semen requires closure of the bladder neck and rhythmic contractions of the urethra and perineal striated muscles. Behavioural characterisation of ejaculation is apparent when the male maintains the mounting position for a brief period (1-2 secs) after the last series of intromissions; following this the female initiates a separation. The male will then tend to groom his genital region afterwards and engage in a brief period (4-6 min) of sexual inactivity. As seen with central control of penile erection, the spinal cord serves as the primary central site controlling ejaculation via the spinal ejaculation generator (Mckenna, 1999) which influences autonomic and somatic nuclei in the lumbosacral spinal cord (Allard et al, 2005). This was implied after studies showing that removal of descending supraspinal influences after spinal cord injury does not attenuate ejaculatory responses to peripheral stimulation in humans and rats (Brackett, 1999; Griffith et al, 1973; McKenna et al, 1991; Pescatori et al, 1993). Thus, the spinal cord appears to be the main central controller of the ejaculatory response. Sympathetic inputs influencing ejaculation reside in the thoracolumbar segments of the spinal cord (Baron and Janig, 1991; Nadelhaft and McKenna, 1987). Conversely, those exerting parasympathetic control originate in the sacral parasympathetic nucleus (SPN) of the lumbosacral spinal cord (Hancock and Peveta, 1979a, 1979b; Nadelhaft and Booth, 1984). During ejaculation, in addition to a coordinated sympathetic and parasympathetic outflow from the spinal ejaculation generator, there is also

convergent somatic output from the pudendal motorneurons innervating the perineal striated muscles (Coolen et al, 2004 for review). Rhythmic activation of these motorneurons facilitates the expulsion of semen and contributes to the heightened pleasurable sensations experienced during ejaculation (Gerstenberg et al, 1990; Holmes et al, 1991; Holmes and Sachs, 1991). The neural substrates and circuitries subserving ejaculation still remain poorly understood. However, recently there has been some progress in the elucidation of these pathways as shown by Truitt and Coolen (2002) who demonstrated that a specific set of lumbar spinothalamic cells located in the L3-L4 spinal cord region are a component of the spinal ejaculation generator and are crucial for eliciting the ejaculatory response (Coolen et al, 2004; Truitt and Coolen, 2002). In rats, the relaying of sensory information during ejaculation is believed to be conveyed by the dorsal penile nerve and the pudendal nerve (McKenna et al, 1991; Pescatori et al, 1993).

Thus, it can be seen that the consummatory elements of male sexual behaviour is comprised of three phases; penile erection, intromission and ejaculation (often defined as copulatory behaviour). There is abundant data on the central processes and neural correlates involved in the expression of penile erection in particular and because this sexual component precedes intromission and ejaculation and acts as a physiological stimulator for both, it is necessary to better understand neuroanatomically and neurochemically those central pathways activated during erectile function. We know that the spinal cord is the central mediator for penile erection; however, it is still unclear as to those supraspinal influences that are able to modulate neurotransmission within the pro-erectile spinal networks.

1.5 Brain nuclei involved in the central control of penile erection.

The significant role of the brain in mediating penile erection was first shown by Benassi-Benelli and colleagues (1979) who found that pharmacologically-induced penile erection (agonist given systemically) could be inhibited by a centrally- but not a peripherally-acting antagonist. This finding has since been confirmed in other studies (Ferrari and Claudi, 1991; Hsieh et al, 2003; Pehek et al, 1988) and has reinforced the notion that central brain mechanisms partly regulate erectile function. Several brain structures have been implicated in the neural control of penile erection, with the hypothalamic nuclei showing the most convincing evidence for a prominent role. However, it is

important to add that there are several studies which show that central influences on penile erection are not restricted to the hypothalamus but involve other subcortical structures (Summary diagram is shown in Figure 1.2).

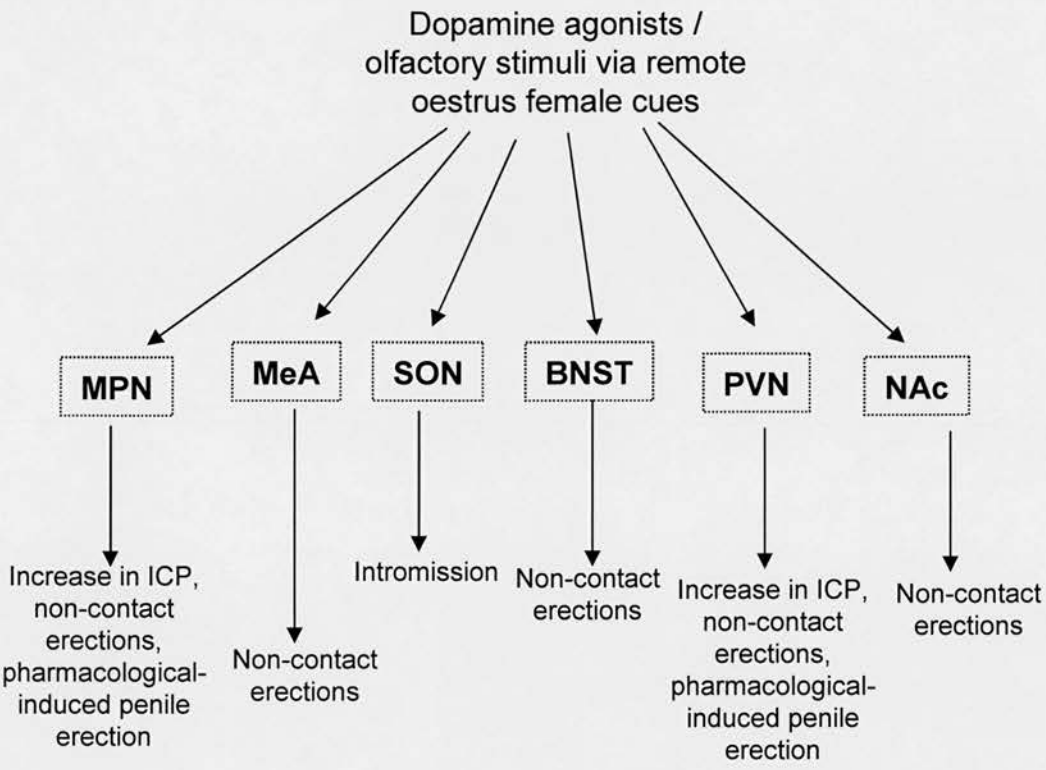
1.5.1. Hypothalamus

It is widely accepted that the hypothalamus serves as a major integration site, where central, sensory and peripheral inputs converge to regulate various neuroendocrine-mediated behaviours. Specific nuclei within the hypothalamus are believed to be important sites for regulating erectile function, namely the medial preoptic area (MPOA), the paraventriclar nucleus (PVN) and to a lesser extent the supraoptic nucleus (SON).

MPOA

The MPOA (containing the MPN) (Figure 1.3A) has proved to be an important integration site for the expression of male sexual behaviour. This was demonstrated after lesioning studies of the MPOA showed almost complete abolishment of male copulatory function in various species (Koyama et al, 1984; Liu et al, 1997; Lloyd and Dixson, 1988). With regard to the role of the MPOA during penile erection, there have been several conflicting reports questioning the importance of the MPOA in eliciting erectile events. Using intracavernous pressure (ICP) as an indicator of erectile function, there have been several studies demonstrating that electrical stimulation of the MPOA produces powerful transient increases in ICP (Giuliano et al, 1996; Giuliano et al, 1997; Sato and Christ, 2000). Additionally, by altering endogenous neurotransmitter levels in the MPOA, non-contact and reflexive erections can be augmented (Adachi et al, 2003). Immunocytochemical studies using *Fos* as a marker of neuronal activation have indicated that upon exposure to oestrus female cues, non-contact erections, copulation and ejaculation have all been shown separately to increase neuronal activation in the MPOA of male rats (Bressler and Baum, 1996; Cooleen et al, 1996; Robertson et al, 1991; Shimura et al, 1994; Struthers, 2001; Veening and Coolen, 1998). However, support for a role of the MPOA in the central control of penile erection was diminished after lesioning studies showed that damage to the MPOA and its fibres had no significant effects on non-contact erections but severely disrupted copulatory behaviour (Liu et al, 1997). Pharmacological studies in involving microinjection

Figure 1.2: Summary diagram of central structures involved in penile erection



Summary of those nuclei implicated in central control of penile erection. Both the MPOA and PVN appear to be key players in initiating penile erection in various contexts. The SON may have a role during *in-copula* penile erection. The MeA, BNST and NA are necessary for the integration of sexual stimuli with sexual arousal processes. MPN=medial preoptic nucleus; MeA=medial amygdala; SON=supraoptic nucleus; BNST=bed nucleus of stria terminalis; PVN=paraventricular nucleus; NAc=nucleus accumbens.

of dopaminergic ligands directly into the MPOA (primary site for mediating the dopaminergic effects on copulation) (Hull et al, 1986; Pehek et al, 1988) have similarly shown conflicting reports. Injection of the dopamine agonist, quinelorane into the MPOA (1μg) had a facilitatory role in erectile function (Bazzett et al, 1991; Markowski et al, 1994). Conversely, in another study where quinelorane was injected into the MPOA (2.5μg), erectile function was inhibited and yet seminal emission enhanced (Bitran et al, 1989). However, pharmacological data should be interpreted with caution as the erectile responses appear to depend on which MPOA receptors that are stimulated by the exogenously applied pro-erectile drug and the dose administered (Hull et al, 1992; Markowski et al, 1994).

Thus, it seems that the MPOA is crucial for efficient copulatory behaviour in rats. Because removal of MPOA influence on erectile pathways has only a minor impact on penile erection; its role in the initiation of penile erection appears to be modulatory. It is very likely that MPOA involvement in erectile function is indirect via MPOA projections to another important pro-erectile structure, the PVN (Conrad and Pfaff, 1976).

PVN

The PVN (Figure 1.3B) is believed by some researchers to be critical for the initiation of penile erection upon pharmacological stimulation or exposure to oestrous female cues (Argiolas and Melis, 2004; Melis et al, 1998, 1999). The PVN proves to be a suitable candidate because it receives neural inputs from almost all hypothalamic nuclei and its branching anatomical arrangement allows for discrete action at multiple target sites in the CNS. The PVN is comprised of five subregions, namely, the anterior, dorsal, medial, magnocellular and lateral subdivisions (Figure 4). Because specific parvocellular neuronal groups have been implicated in mediating penile erection (Kita et al, 2006, Witt and Insel, 1994), there has been some suggestion of a paraventricular subregion-specific activation during erection. Parvocellular neurons located in the PVN have been shown to project to the lumbosacral spinal cord and lie in close apposition to spinal pro-erectile networks (Tang et al, 1998; Veronneau-Longueville et al, 1999). Thus, such a defined neuroanatomical pathway could serve as one potential route through which supraspinal commands are transmitted to the erectile centres in the spinal cord. Electrical stimulation of the PVN and micro-injection of various

pharmacological agents including those that mimic the actions of dopamine, oxytocin, nitric oxide, excitatory amino acids into the PVN have all been shown to have profound facilitatory effects on penile erection (Argiolas and Melis, 2005 for review; Chen et al, 1997; Melis et al, 1987). Neural mapping studies using *Fos* as a marker of neuronal activation, have shown the PVN to be activated during various aspects of masculine sexual behaviour (Caquineau et al, 2006; Kita et al, 2006; Pfaus and Heeb, 1997; Witt et Insel, 1994). Additionally, experiments involving lesioning of either part or all of the PVN show that there are significant deficits in penile erection (Paredes and Agmo, 2004) which do not appear to be due to a direct effect on penile striated muscles as PVN-derived parvocellular fibres innervating somatic outflow are very sparse (Tang et al, 1999). Chemical destruction of the parvocellular PVN using the excitotoxin, NMDA, which leaves predominantly the magnocellular region intact in the PVN shows there are significant deficits in erectile activity as evidenced by an increase in the latency to achievement of penile erection and a marked decrease in the frequency of erections (Liu et al, 1997). Moreover, such PVN lesions completely abolish the pro-erectile effect of the nonselective dopamine agonist, apomorphine and oxytocin (Argiolas et al, 1987). These results suggest that the parvocellular PVN neurons (namely, anterior, dorsal and medial fibres known to project to the spinal cord) (Wagner and Clemens, 1991) may have an intermediary role in the excitation phase of penile erection and are perhaps crucial during sexual arousal and the initiation of erection. In contrast, the magocellular subdivision of the PVN appears to have a role in the consummatory elements of sexual behaviour (eg. intromission, pelvic thrusting, ejaculation) (Caquineau et al, 2006; Pattij et al, 2005). Since the PVN receives diffuse innervation from some hypothalamic and extra-hypothalamic structures that have been implicated in penile erection (Gray et al 1989; Herman et al, 2005; Liposits, 1993; Larsen et al, 1994; Prewitt and Herman, 1998) and its projections impinge on spinal pro-erectile centres (Veronneau-Longueville et al, 1999), it seems logical to assume that the PVN may serve as the final common nucleus which supplies descending excitatory fibres to the lumbosacral spinal cord. Thus, the PVN may have a more pivotal role in triggering the expression of penile erection.

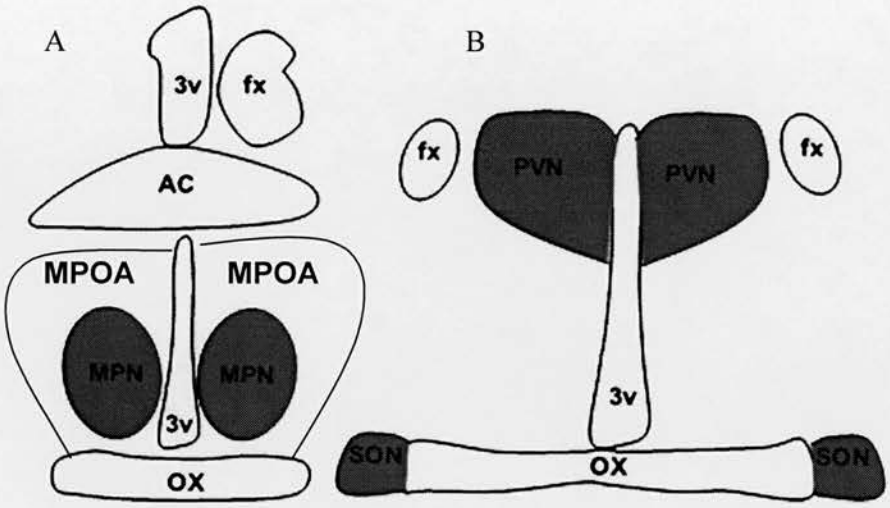


Figure 1.3: Diagrammatic representation of rat brain on coronal view. Hypothalamic nuclei involved in the central control of penile erection are the MPN (A), SON and PVN (B). 3v=third ventricle; fx=fornix; AC=anterior commisure; MPOA=medial preoptic area; MPN=medial preoptic nucleus; OX=optic chiasm; SON=supraoptic nucleus; PVN=paraventricular nucleus.

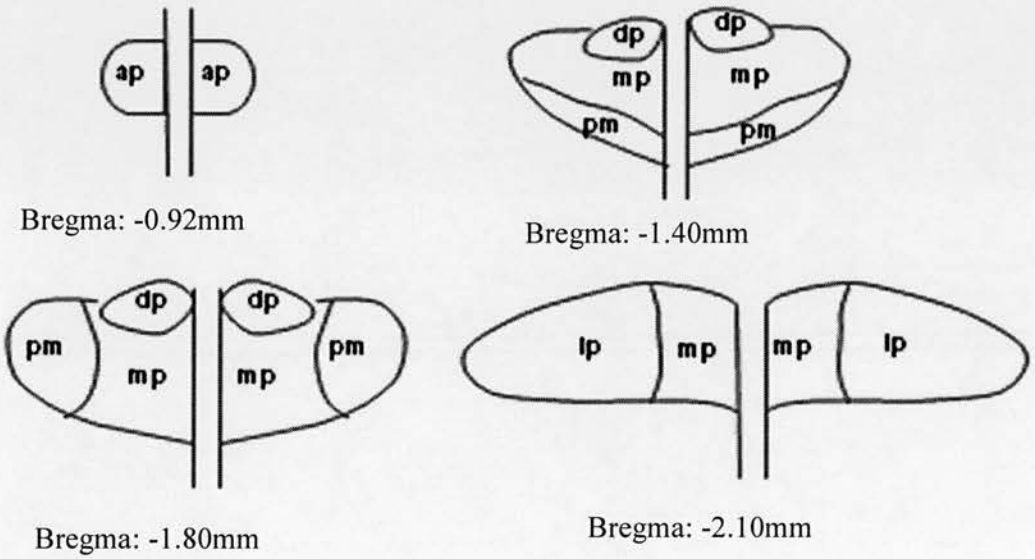


Figure 1.4: Subdivisions comprising the PVN of the rat, represented in coronal view. Based on the atlas of Paxinos and Watson, 1988. ap=anterior parvocellular; dp=dorsal parvocellular; mp=medial parvocellular; lp=lateral parvocellular; pm=posterior magnocellular.

SON

Relatively little is known regarding the role of the predominantly magnocellular SON (Figure 1.3B) in central control of penile erection. Fos immunocytochemical studies have revealed that *in-copula* penile erection (intromission) and ejaculation produced dense clusters of Fos expression in the SON (Caquineau et al, 2006; Pattij et al, 2005; Phillips-Farfan and Fernandez-Guasti, 2007). Interestingly, in males deemed sexually sluggish, there is very weak neuronal activation in the SON (Pattij et al, 2005). So, there appears to be emerging evidence to suggest that this nucleus may be activated during the display of copulatory behaviour.

1.5.2 Medial amygdala (MeA)

The basolateral and corticomedial amygdala are believed to be involved in the processing of sexually conditioned odours and have a role in sexual reward (Kippin et al 2003; Lehman and Winans, 1982). However, it is the MeA that has shown to exert some control of the erectile process. The MeA is typically known to be involved in social recognition and social interaction (Ferguson et al, 2001; Ferguson et al, 2002 for review). In addition, there is some support for an established role of the MeA in the mediation of male sexual behaviour (Harris and Sach, 1975; Kondo et al, 1992). Because it receives major olfactory imputs, the MeA is regarded as an important integrative site for mediating sexually-stimulating cues and the evocation of penile erection. Robinson and Mishkin (1968) initially demonstrated that electrical stimulation of the MeA in primates had a powerful effect on penile erection. More recently, it has been shown that male rats with damage to the MeA display impairments in non contact erections but no obvious deficits in reflexive erections or copulation were observed (Kondo et al, 1998; Kondo and Sachs, 2002). Thus, the MeA appears to be another important central candidate that is part of a larger network regulating erectile function.

1.5.3 Bed nucleus of the stria terminalis (BNST)

The BNST is another brain region which has been implicated in the neural control of non-contact erections. As seen with the PVN and MeA, destruction of the BNST has profound inihibitory effects on the expression of penile erection in response to female oestrous cues, whilst producing no apparent deficits in full copulatory function (Kondo et al, 1998; Liu et al, 1997). Reciprocal connections exist

between the BNST and the MeA, MPOA and PVN (Canteras et al, 1995; Dong and Swanson, 2006; Saphier and Feldman, 1986; Sawchenko and Swanson, 1983), thus the BNST seems to contribute in part to the neural circuitry subserving penile erection.

1.5.4 Nucleus accumbens (NAc)

The nucleus accumbens (NAc) has been shown to have some influence on a diverse range of behaviour including fear and social isolation (Fulford and Marsden, 1998; Lapiz et al, 2001 for review; Schwienbacher et al, 2003). The NAc also has a key central role in the sexual reward circuit as evidenced by an increase in NAc neuronal firing and intra-NAc monoaminergic neurotransmission when pairing olfactory stimulation with sexually receptive oestrus females (Damsma et al, 1992; West et al, 1992). However, its role in regulating masculine sexual behaviour is less well defined. The NAc is known for its role in sexual motivation. Pharmacological agonists microinjected into the NAc which act to mimic or enhance dopamine release, were shown to reduce the time to which male rats displayed mounting and intromitting compared to control rats (Hull et al, 1986; Everitt et al, 1990; Giuliano and Allard, 2001). Additionally, local infusion of the dopamine antagonist, haloperidol into the NAc, decreased sexual motivational parameters (Pfaus and Phillips, 1991). The importance of the NAc in mediating the comsummatory elements of sexual behaviour is less well defined. Neurotoxic and electrolytic disruption of the NAc has shown that the fundamental aspects of male sexual behaviour remain intact (Liu et al, 1998); however, when exposed to a receptive yet inaccessible female, substantial deficits in the display of non-contact erections were observed (Kippin et al, 2004; Liu et al, 1998). The NAc appears to have some role in responding to remote sexual cues and integrating such stimuli with those processes controlling sexual arousal.

Thus, it can be seen that central control of penile erection involves a complex and hierarchical neuronal network operating at the supraspinal level which subsequently influences the spinal erectile network. However, neurochemical substrates and neuronal phenotypes that may modulate central neurotransmission remain to be fully elucidated.

1.6. Oxytocin

Oxytocin is a classical neuroendocrine hormone that can initiate a wide spectrum of peripheral and central effects. It is a nonapeptide comprised of nine amino acids and it is very similar in structure to the peptide hormone, arginine vasopressin (AVP), where it differs in only two of the nine amino acid sequences.

1.6.1.1 Oxytocin synthesis and distirbution

Oxytocin is primarily synthesised in magnocellular neurosecretory cells in the SON and PVN, where it is transported to the posterior pituitary and released into the blood. From here oxytocin has a vital role in mediating parturition and the milk ejection reflex in rats (Insel et al, 1997b; Neumann, 2001a; Russell et al, 2001; Russell et al, 2003 for review). Similarly, oxytocin is also generated in parvocellular neurons in the PVN which project to extrahypothalamic regions within the CNS where they have a role in mediating various autonomic functions (Badoer, 2001; Mack et al, 2002; Petersson, 2002; Tang et al, 1998). Oxytocinergic fibres are not restricted to the SON and PVN but also lie outwith these two nuclei where they are found to be expressed in various other brain nuclei including the hippocampus, cortex, amygdala, olfactory bulbs, substantia nigra, brain stem and the spinal cord (Gimpl and Fahrenholz, 2001 for review and references therein; Sawchenko and Swanson, 1985; Sofroniew and Weindl, 1978). With its diffuse projections, oxytocin is able to influence a range of neuroendocrine-mediated functions governing social and affiliative behaviours such as maternal and socio-sexual behaviour (Argiolas and Melis 1995; Insel et al., 1997a, b; Argiolas, 1999; Argiolas and Melis 2004; Kendrick, 2000, 2004; Richard et al, 1991 for review).

1.6.1.2 Oxytocin release

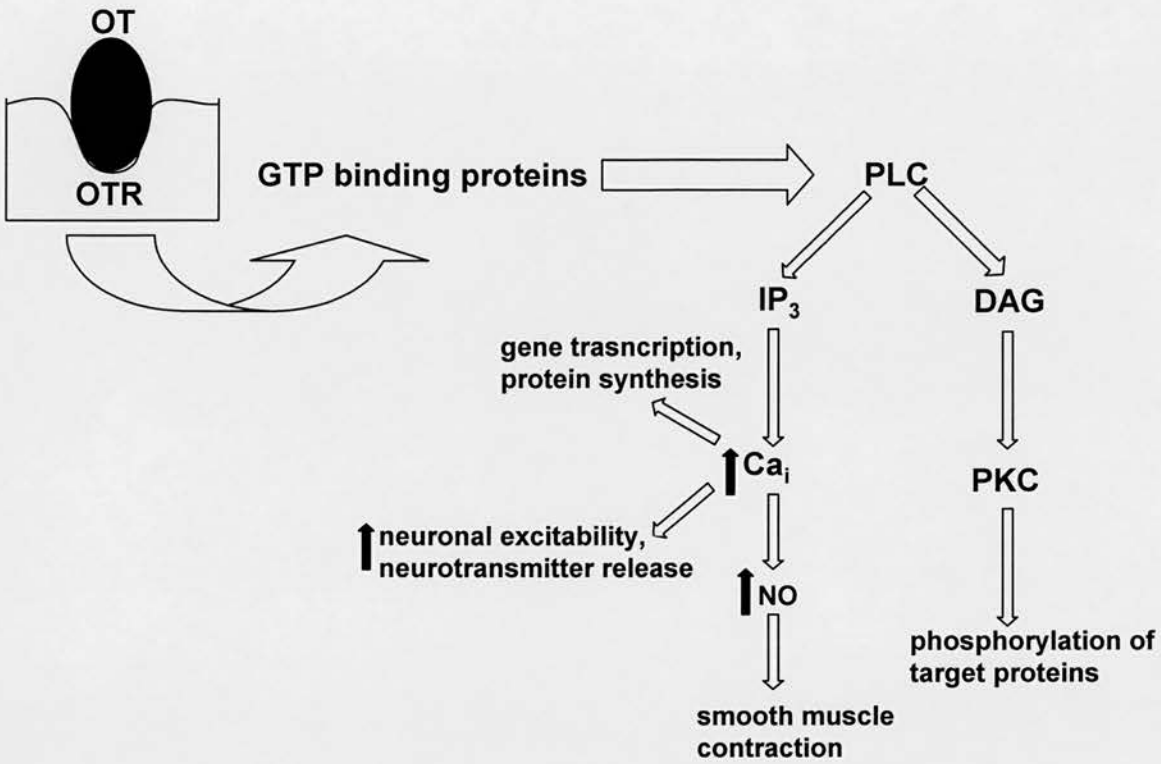
As previously mentioned, magnocellular and parvocellular oxytocn release into systemic circulation and the CNS occurs via projections to the posterior pituitary and extrahypothalamic brain regions, respectively. Oxytocin release from axon terminals occurs in the classical manner where axonal release is preceeded by an influx of calcium into axonal terminals in response to an invading action potential. However, as first demonstrated by Moos and colleagues (1984), oxytocin can also be released somatodendritically from magnocellular oxytocin neurons in the PVN and SON to regulate

its own release. This finding was further substantiated in numerous in vivo studies using microdialysis to quantitatively measure oxytocin release in the plasma and the brain of parturient and lactating rats (Moos et al, 1989; Neumann et al, 1993a; Neumann et al, 1993b). Unlike axonal release of oxytocin, dendritic release of oxytocin is triggered by release of calcium from intracellular stores and is generally electrically-independent (Ludwig et al, 2002b; Ludwig and leng, 2006 for review). Central (somatodendritic) and peripheral (axonal) oxytocin release from magnocellular cells can act synergistically to influence behavioural consequences. During suckling, there is a concomitant release of oxytocin into the bloodstream, SON and PVN which contributes to the milk ejection reflex (Moos et al, 1989; Neumann et al, 1993b). This simultaneous release of oxytocin into the brain and periphery was also observed in female (intruder) rats during an emotional stressor when a virgin intruder is introduced to the homecage of and confronted with a lactating female rat (Bosch et al, 2004; Neumann et al, 2001b). Such synergy between the central and peripheral oxytocin systems does not always exist and infact there can be an apparent disassociation between the two as seen during another psychosocial stressor such as social defeat. Engelmann and colleagues (1999) demonstrated that whilst intra-SON oxytocin release increased in response to social defeat, peripheral oxytocin release remained unaffected. Thus, it can be seen that during certain neuroendocrine-mediated behaviours, centrally-acting and peripherally-acting oxytocin may act in unison or independently to exert their behaviourally-specific effects.

1.6.1.3 Oxytocin receptors

The encoded oxytocin receptor is a 389-amino acid polypeptide with seven transmembrane domains and is thus part of the G protein-coupled receptor family. When oxytocin binds to its receptor it initiates a cascade of intracellular events that culminate in a range of cellular responses including an increase in neuronal firing, neurotransmitter release, smooth muscle contraction and protein phosphorylation (Figure 1.5). In rats, peripheral expression of oxytocin receptors is concentrated in the male and female reproductive tract and in myoepithelial cells in mammary tissue (Gimpl and Fahrenholz, 2001; Zhang et al, 2005). Additionally, oxytocin receptors are also abundantly expressed throughout the CNS and often exist in the same regions expressing oxytocin fibres. In addition to their expression in the SON and PVN, oxytocin receptors are also found in the regions of the cortex,

Figure 1.5 : Oxytocin intracellular signalling pathways



Transduction pathways activated upon oxytocin receptor stimulation. Oxytocin receptors are G protein-coupled receptors . Oxytocin binds to an oxytocin receptor coupled to GTP binding proteins which in turn activate PLC. From there, PLC stimulates DAG which generates PKC and results in the phosphorylation of target proteins. Additionally, PLC activates IP_3 which triggers a cascade of calcium dependent mechanisms which promote protein synthesis, neurotransmitter release and smooth muscle contraction. OT=oxytocin; OTR=oxytocin receptor; GTP=guanine triphosphate; PLC=phospholipase C; IP_3=inositol triphosphate; DAG= diacylglycerol; Ca_i =intracellular calcium; PKC=protein kinase C; NO=nitric oxide.

hippocampus, limbic system, basal ganglia, medial preoptic area, olfactory bulbs, amygdala and the brain stem (Freund-Mercier et al, 1994; Gimpl and Fahrenholz, 2001; Yoshimura et al, 1993). There is widespread distribution of oxytocin receptors in the thoracic and lumbosacral segments of the spinal cord, with the dorsal horn, dorsal grey commisure, intermediolateral cell column all possessing oxytocin receptors (Veronneau-Longueville et al, 1999). However, some brain areas show a distinct mismatch between oxytocin fibre distribution and oxytocin receptor expression, such as seen in the amygdala and olfactory bulbs where there is a significantly greater proportion of oxytocin receptors compared to oxytocin fibres that innervate these nuclei (Ferguson et al, 2001; Huber et al, 2005; Terenzi and Ingram, 2005). Such an anatomical mismatch gives rise to the possibility that centrally released oxytocin can diffuse to distant sites within the brain to exert its effects. The oxytocinergic system may also be involved in complex interactions with other neuromediatory pathways which regulate oxytocinergic and non-oxytocinergic neurotransmission in the brain. Such a finding was recently seen in a study examining the occurrence of penile erection in conscious male rats where local injection of oxytocin into the ventral tegmental area stimulated release of dopamine in the nucleus accumbens and the PVN (Melis et al, 2007).

1.6.1.4 Oxytocin and behaviour

In the periphery, oxytocin is widely known to have a potent stimulatory effect on uterine muscle tone and triggering the onset of labour (Fuchs et al, 1995; Kimura et al, 1992). In males, the testis and the epididymis are peripheral targets of oxytocin where it aids in maintaining spermiation and sperm motility (Gimpl and Fahrenholz, 2001 for review). In the brain, the actions of oxytocin have been shown to be important in regulating socio-sexual behaviours. Oxytocin pathways subserving maternal and social behaviour are believed to be important in governing familial and non-familial bonds (Campbell, 2008 for review; Cushing and carter, 2000; Ferguson et al, 2000; Kendrick, 2000). In monogamous prairie voles, centrally delivered oxytocin markedly increases the length of time spent in social contact, implying that oxytocin facilitates pair bond formation within this strain of voles (Cho et al, 1999). Finally, central oxytocin has also been shown to have anxiolytic properties whereby oxytocin-treated mice engage in more risk-taking, explorative and investigative behaviours (Bale et al, 2001; McCarthy et al, 1996; Ring et al, 2006).

1.6.2 Oxytocin and male sexual behaviour

The role of oxytocin in male sexual behaviour has been well documented (Argiolas and Melis, 2004). It has long been known that oxytocin when injected either peripherally, centrally or intra-thecally, can be an extremely potent inducer of penile erection (Argiolas et al, 1985; Argiolas et al, 1986; Martino et al, 2005; Kita et al, 2006) and has a strong pro-copulatory effect in male rats (Arletti et al, 1985). In humans, plasma oxytocin levels are significantly elevated during arousal, penile erection and ejaculation (Uckert et al, 2003; Carmichael et al, 1987). Both rodent and human studies suggest that oxytocin acts as an important mediator for appetitive and consummatory phases in male sexual behaviour, serving as a facilitatory neuromodulator during sexual arousal and consummation. The medial preoptic nucleus (MPN) of the MPOA, SON and PVN are three key hypothalamic nuclei believed to partly influence male sexual behaviour via their oxytocinergic contributions. The PVN and SON are the richest oxytocin-containing nuclei in the brain whereas the MPN of the MPOA expresses a comparatively small oxytocinergic neuronal population. Pharmacological and neuroanatomical studies have shown that specific facilitatory effects on sexual behaviour are partly modulated via oxytocin action within these structures. Thus, these three nuclei are potential key candidates for neural control of erectile function.

1.6.2.1 MPOA

Currently, all sexual behaviour studies implicating oxytocin action in the MPOA have been performed on female rodents. To our knowledge, no such neuropeptide involvement in the MPOA has been investigated in male rats. Central infusion of increasing doses of oxytocin dramatically increases female soliciting behaviours, such as the lordortic response in ovariectomised, hormonally-primed female rats and hamsters (Caldwell et al, 1989; Whitman and Albers, 1995; Schulze and Gorzalka, 1991). Likewise, oestrogen-primed females injected with an oxytocin receptor antagonist in the MPOA show a reduction in the expression of lordosis postures (Caldwell et al, 1994). Furthermore, immunocytochemical studies revealed that mounting by male rats increased oxytocin immunoreactivity in the MPOA of female rats (Caldwell et al, 1989). So, in females the MPOA appears to be a primary site for the facilitative effects of oxytocin to induce sexual receptivity. Thus,

it is entirely possible that oxytocin populations residing in the MPOA may also be similarly effective in eliciting sexual responses in male rats.

1.6.2.2 PVN

Considerably more is known regarding the role of oxytocin in the PVN during penile erection and this has previously been extensively reviewed (Argiolas and Melis, 2004). Recruitment of oxytocin neurons in the PVN during sexual behaviour has recently been substantiated after increased oxytocin release in the PVN was correlated with mating behaviour in male rats (Wadherr and Neumann, 2007) implicating paraventricular oxytocin involvement during copulation. It is widely accepted that oxytocin can be released somatodendritically from magnocellular oxytocin neurons in the PVN (and SON) (Ludwig, 1998), suggesting that local oxytocin release within this nucleus has an autoregulatory effect. Furthermore, it has been demonstrated that somatodendritic release of oxytocin can increase neuronal firing in magnocellular oxytocin neurons (Ludwig, 2002a, Richard et al, 1997). These findings infer that oxytocin can in fact modulate its own activity via direct and/or indirect actions on oxytocin neurons in the PVN (and SON) (Ludwig, 2002). Thus, it is quite possible that such oxytocinergic activation via local oxytocin may underlie the neuroendocrine pathways facilitating penile erection.

Electrolytic and neurotoxic destruction of the oxytocin rich PVN, which results in widespread loss of central oxytocin levels (Hawthorn et al, 1985), abolishes the erectile response to oxytocin and other pharmacological ligands (Argiolas et al; 1987; Liu et al, 1997). Additionally, blockade of central oxytocin receptors after pre-treatment with an oxytocin receptor antagonist disrupts non-contact erections (Melis et al, 1999) and penile erections evoked after intra-PVN delivery of oxytocin (Argiolas, 1999 for review; Melis and Argiolas, 2003).

Immunocytochemical studies using combined Fos and oxytocin labelling as a marker of oxytocin neuronal activation have provided additional neuronatomical support of an underlying oxytocinergic pathway mediating penile erection and copulation. An increased Fos expression in oxytocin neurons was observed in lateral parvocellular oxytocin neurons in the PVN during intromissions and

ejaculations (Witt et al, 1994). Furthermore, Fos expression in oxytocin neurons increased with the intensity of sexual stimuli (ranging from an oestrus odour to intromission and ejaculation) (Witt et al, 1994). Oxytocin-induced penile erection was shown to selectively activate paraventricular parvocellular oxytocin neurons known to project to the spinal cord (Kita et al, 2006). Meanwhile, paraventricular magnocellular oxytocin cells were also shown to be stimulated during intromission (Caquineau et al, 2006).

Finally, electrical and tactile stimulation of the dorsal penile nerve, selectively excites oxytocin cells in the PVN (Yanagimoto et al, 1996) implying that sensory afferents arising from the penis have a role in peripheral feedback.

Thus, it seems that the PVN is a crucial brain area necessary for responding to the pro-erectile effects of oxytocin.

It is not clear whether local oxytocin release (presumably via magnocellular oxytocin neurons) or distant oxytocin release (via parvocellular oxytocin neuronal projections) function sequentially or in concert to help coordinate the erectile response. Additionally, the complexity of oxytocin involvement is enhanced by the presence of both a parvocellular and magnocellular neuronal population, which some believe may differentially influence aspects of masculine sexual behaviour. In all likelihood, it is very possible that dendritic release of oxytocin within the PVN activates both oxytocin neuronal phenotypes which in turn cause central release of oxytocin at multiple sites. It is this central wide presence of oxytocin that presumably initiates the rapid onset of penile rigidity and facilitates the consummatory parameters that follow during mating behaviour, although this is highly specualative.

1.6.2.3 SON

Very little is known regarding the role of the magnocellular oxytocin system in the SON during penile erection. Similar to the PVN, magnocellular oxytocin neurons exist in the SON where they too project to the posterior pituitary and are secreted into the blood to exert their peripheral hormonal actions (Kiss and Mikkelson, 2005 for review). The potential role of magnocellular oxytocin cells in the regulation of erectile function has been poorly evaluated; however, human studies have revealed

that plasma oxytocin levels only show a marked increase after ejaculation and not during sexual arousal (Murphy et al, 1987). In another study, where men were exposed to erotic stimuli, only a marginal increase in plasma oxytocin levels was observed during the flaccid to semi-erect state of the penis. However, no such changes in neuropeptide concentration occurred following that when penile rigidity was achieved. Conversely, once the penis returned to the flaccid state, there was a steady increase in systemic plasma oxytocin levels (Uckert et al, 2003). The sexually-specific increase in systemic oxytocin levels after ejaculation has also been confirmed in animal models (Stoneham et al, 1985). These findings postulate that magnocellular oxytocin release into the systemic circulation is not essentially involved in penile erection associated with sexual arousal but may have an underlying functional role in signalling the end of a sexual encounter and the beginning of sexual satiety. It is important to add that whilst magnocellular oxytocin cells have a clear peripheral role in mediating various sexual responses that currently do not include the initiation of penile erection, a central pro-erectile role (via local somatodendritic release) in the SON cannot be ruled out and merits further investigation.

The effect of intra-SON injection of oxytocin or other pro-erectile drugs on penile erection is not known. However, in support of the oxytocin release data, immunocytochemical studies have revealed that supraoptic oxytocin cells become activated during intromission (Caquineau et al, 2006) and ejaculation (Pattij et al, 2005) in rats. Thus, it seems that supraoptic oxytocin cells are stimulated upon *in-copula* penile erection (after achievement of penile rigidity) and ejaculation. As with the PVN, stimulation of the dorsal penile nerve excites oxytocin cells in the SON (Honda et al 1999) suggesting they too may be highly responsive to peripheral sensory afferents originating in the penis.

1.7 Control of oxytocin neurons during penile erection

It is apparent that central oxytocinergic neurons have an established role in mediating erectile function; however, those neural circuits impinging on and modulating the activity of oxytocin neurons during the expression of masculine sexual behaviour are poorly understood. Many central factors such as the serotonin, melanocortin, glutamate and GABA systems are all believed to have a part in neuromodulatory influence on oxytocinergic neurotransmission (Argiolas and Melis et al, 2004 for

review; Caquineau et al, 2006; De Jong et al, 2007; Martin and MacIntyre, 2004; Melis et al, 2006; Pederson and Boccia, 2006; Succu et al, 2006).

1.7.1 Noradrenlalin and oxytocin interactions

To our knowledge, there does not seem to be any published work showing a link between central noradrenaline and oxytocin during penile erection. Noradrenaline is a neuromediator for the sympathetic (anti-erectile) nervous system, so it seems unlikely that it would have a strong facilitatory influence over penile erection Hippocampal noradrenergic neurotransmission is believed to play a role in the negative feedback loop controlling penile erection (Chang et al, 2001) but a central role in facilitating erectile function has not been established (Martinez-Pineiro et al, 1994). In hormonally-primed female rats, oxytocin has been shown to stimulate noradrenaline release in the ventromedial hypothalamus during lordosis (sexually-receptive behaviour) (Etgen and Karkanias, 1994; Vincent and Etgen, 1993). However, such a link has yet to be investigated in male rats during masculine sexual behaviour.

1.7.2 Serotonin and oxytocin interactions

There is emerging evidence for an interactive role between serotonin and oxytocin during ejaculation which has previously been extensively reviewed (De Jong et al, 2006, 2007 for review). Additionally, there has been some published data suggesting a link between these two neuromodulators during the expression of penile erection. Stimulation of central serotonin receptors (particularly the 5HT-1C subtype) elicits penile erection and this has been correlated with elevated plasma oxytocin levels (Bagdy et al, 1992; Bagdy and Kalogeras, 1993). Neuroanatomical studies have also revealed that in addition to oxytocin projections, there is a distinct serotonerigc innervation of the lumbosacral spinal cord where serotonin fibres are believed to impinge on preganglionic fibres of the spinal pro-erectile network (Tang et al, 1998). Contained within this spinal nucleus are oxytocin receptor binding sites (Veronneau-Longueville et al, 1999) suggesting that the lumbosacral spinal cord may serve as another central integration site for serotonin and oxytocin neurotransmission.

The involvement of the oxytocin-rich PVN in mediating serotonin responses however, remains debatable after conflicting findings. Destruction of the PVN did not significantly impair penile erection or affect plasma oxytocin levels after central administration of 5HT-2C receptor agonists (Bagdy and Makara, 1995). However, these findings conflict with other studies which showed 5HT-2C agonists were able to enhance oxytocin concentrations in plasma (Bagdy and Makara, 1994; Bagdy, 1996), although erectile events were not recorded in these studies. In these experiments, the serotonin agonists were administered into the lateral ventricle, so perhaps they were acting at oxytocin sites other than the PVN to produce the differential oxytocin responses observed in the above studies. Taken together, the published data highlights a possible serotonin-oxytocin link during penile erection and suggests that integration sites may exist not only in the brain but at the level of the lumbosacral spinal cord, which is crucial for inducing erectile episodes.

1.7.3 Melanocortin and oxytocin interactions

Melanocortins such as melanotan-II (MT-II) and alpha-melanocyte stimulating hormone (α-MSH) are known to act on all subtypes of melanocortin receptors (Martin and MacIntyre, 2004) and have been shown to be potent erectogenic agents in animals and humans (Bertolini and Gessa, 1981; Wessells et al, 1998). Because melanocortin receptors are abundantly expressed in the MPN, SON and PVN (and many other hypothalamic and extra-hypothalamic regions) (Adan and Gispen, 1997), it is possible that melanocortins may be involved in the regulation of central oxytocin responses. Although it is presumed that melanocortin and oxytocin interactions are likely to occur in hypothalamic nuclei to mediate erectogenesis, this was shown not to be the case based in studies where the pro-erectile effects of peripheral but not central administration of melanocortins were blocked after oxytocin receptor blockade (Martin et al, 2002; Mizusawa et al, 2002). This suggests that melanocortins may act indirectly on central oxytocin sites or via non-oxytocinergic targets. However, a more direct role for melanocortin influence over central oxytocinergic transmission was suggested by immunocytochemical studies where central delivery of α-MSH increased the activation of magnocellular oxytocin neurons in the SON and PVN. Furthermore, central injection of a melanocortin receptor antagonist (which selectively targets the melanocortin receptor 4, or MC4) significantly attenuated the activation of magnocellular oxytocin neurons. Although this antagonist

did not have a significant inhibitory effect on the expression of erectile function in this study, rats did take longer to display penile erection indicating some impairment of erectile parameters (Caquineau et al, 2006).

1.7.4 Glutamate and oxytocin interactions

The agonist, NMDA, mimics the effects of endogenous glutamate via action on NMDA receptors and enhancing excitatory amino acid neurotransmission. When injected into the PVN, the pro-erectile effects of NMDA (Argiolas, 1999; Melis et al, 1994; Melis and Argiolas, 2003) are believed to involve the stimulation of hypothalamic oxytocin cells as glutamatergic fibres are known to lie in close apposition with paraventricular oxytocin cells (van den Pol, 1991). The contributory role of oxytocin during NMDA-mediated erection was also emphasised after central administration of an oxytocin receptor antagonist blocked NMDA-induced penile erection (Melis et al, 1994). To add to these findings, it has recently been demonstrated that during non-contact erection and copulation, there is marked increase in glutamic acid concentrations within the PVN (Melis et al, 2004), an effect which is believed to be modulated via endocannabinoids (Melis et al, 2006; Succu et al, 2006). Thus, such increased activity levels of glutamate in the PVN specifically during the expression sexual behaviour, make it highly likely that glutamate may exert some excitatory influence over oxytocin neurons to regulate masculine sexual behaviour.

1.7.5 GABA and oxytocin interactions

In addition to the known excitatory neuromodulators, the inhibitory neurotransmitter, GABA has been hypothesised as having an inhibitory role on the generation of penile erection via its GABAergic inputs to hypothalamic oxytocin cells. GABAergic fibres innervate magnocellular oxytocin neurons in the PVN (Jourdain et al, 1999) and almost 50% of all synapses impinging on magnocellular neurons are GABAergic (Gies and Theodosis, 1994). A direct role for GABAergic modulation of central oxytocinergic neurotransmission was established when GABA (applied *in vitro* to SON slices) was shown to influence neuronal firing patterns in magnocellular oxytocin cells (Kombian et al, 1996; Renaud and Bourque, 1991 for review). So, it can be seen that by altering GABAergic neurotransmission, magnocellular oxytocin activity can subsequently be augmented, which in turn

could impact on a range of oxytocin-mediated behaviours such as penile erection. Only relatively recently has the interaction between GABA and oxytocin been looked in the context of penile erection. Melis and Argiolas (2002) found that stimulation of GABA (subtype A) receptors in the PVN was able to inhibit penile erection elicited by oxytocin. Additionally, they were able to reverse this inhibitory effect on erectile function by administering a GABA (subtype A) receptor antagonist. Thus, blocking GABA (subtype A) receptors in the PVN presumably disinhibits magnocellular (and perhaps pavocellular) oxytocin cells. The removal of such inhibitory input would allow oxytocin neurons to respond to excitatory input and so increase oxytocin release and facilitate penile erection. At the moment this is purely hypothetical; however, taken together, the findings lightly touch on the existence of a potential mechanistic pathway that may contribute to evoking penile erection.

1.7.6 Nitric oxide and oxytocin interactions

The peripheral role of nitric oxide (NO) during the expression of penile erection is well researched (Andersson, 2000 for review; Giuliano et al, 1997; Rajfer et al, 1992). In addition, there appears to be an emerging central role for NO in mediating penile erection via paraventricular oxytocin fibres. Penile erection stimulated by intra-PVN injection of NO donors can be antagonised with pre-treatment of an oxytocin receptor antagonist (Melis and Argiolas, 1995). Conversely, injection of inhibitors of nitric oxide synthase (NOS) (the enzyme which generates NO) into the PVN blocks oxytocin-induced penile erection (Argiolas and Melis, 1995; Melis and Argiolas, 1993; Melis et al, 1994). Additionally, the marked increase in paraventricular NO2 and NO3 levels (indicators of NO activity) that occurs concomitant to non-contact erections, was inhibited by an oxytocin receptor antagonist (Melis et al, 2000). Neuroanatomical studies investigating the central expression of penile NOS (PnNOS), a variant of the regulatory protein, neuronal NOS (nNOS) have revealed PnNOS to be abundantly expressed in central nuclei influencing erectile function. PnNOS is generally known to be a peripheral facilitator of the penile framework (Gonzalez-Cadavid et al, 2000). However, it has also been shown to be co-expessed with oxytocin neurons in the MPN, SON and PVN (Ferrini et al, 2003). Both magocellular and parvocellular oxytocin cells were found to be colocalised with PnNOS (Ferrini et al, 2003). Such coexpression of PnNOS and oxytocin indicate that PnNOS may influence oxytocin release into the neurohypophysis (via magnocellular oxytocin neurons) or locally (via somatodendritc

release) to control neuroendocrine-mediated behaviours. Additionally, PnNOS may have a putative role in activating the spinal pro-erectile network via descending parvocellular oxytocin projections. Taken together, these findings implicate NO as an important neuromediator that appears to interact with oxytocin at the supraspinal level (although spinal and peripheral links cannot be ruled out) during male sexual behaviour.

Much of the current research investigating the neural inputs that can alter central oxytocinergic neurotransmission in a penile erection context have focused on the monoamine, dopamine. Dopamine is proving to be one particularly important neurotransmitter that may activate central oxytocin pathways during sexual arousal and copulation (Argiolas and Melis, 2004; Martino et al, 2005; Melis and Argiolas, 2003 for review). Pharmacological, immunocytochemical and release studies have revealed dopamine to have a key facilitatory role in male sexual behaviour.

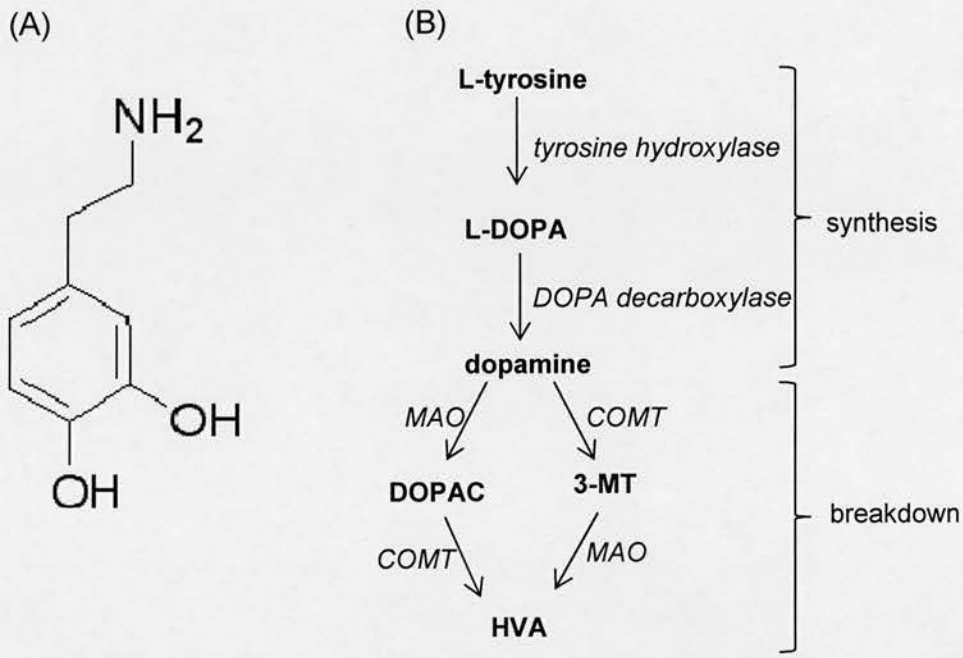
1.8 The role of dopamine and male sexual behaviour

Dopamine is an immensely important central neurotransmitter that has widespread projections and functions throughout the CNS. Dopamine synthesis is a two-step reaction and involves the creation of L-dihydroxyphenylalanine (L-DOPA) from L-tyrosine via tyrosine hydroxylase. L-DOPA is then converted to dopamine by DOPA decarboxylase. Dopamine is then enzymatically converted to 3,4-dihydroxyphenyl acetic acid (DOPAC) and 3-methoxytyramine (3-MT) via the enzymes monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT), respectively. Finally, DOPA and 3-MT are further degraded by COMT and MAO, respectively, to yield the inactive homovanillic acid (HVA) (Figure 1.7).

1.8.1 Dopamine systems

Dopamine has a key role in a range of neurochemical and neurohormonal functions including cognition, sexual behaviour, milk production, arousal, reward, co-ordination and motricity. Dopaminergic neuronal cell bodies originating in the substantia nigra (SN), hypothalamus, ventral tegmental area (VTA), arcuate nucleus and the zona incerta project to various brain structures and comprise six main pathways summarised in table 1.1. Of all the central dopamine pathways, the

Figure 1.7: Dopamine synthesis and breakdown



Synthesis and breakdown of dopamine. The chemical composition of dopamine is illustrated in A. Dopamine is synthesised from the conversion of L-tyrosine to L-DOPA by tyrosine hydroxylase. L-DOPA then becomes dopamine via the actions of DOPA decarboxylase. The breakdown of dopamine involves the actions of the two enzymes, MAO and COMT to produce DOPAC and 3-MT respectively. DOPAC and 3-MT are then subsequnetly degraded to the inactive HVA via COMT and MAO, respectively (B). L-DOPA: L-dihyroxyphenylalanine, MAO: monoamine oxidase, COMT: catechol-O-methyl transferase, DOPAC: 3,4-dihydroxyphenyl acetic acid, 3-MT: 3-methoxytyramine, HVA: homovanillic acid.

Table 1.1: Dopaminergic systems

Dopamine systems	**Origin**	**Projections**	**Function**
Nigrostriatal	SN (A9)	Striatum	Motricity
Mesocortical	VTA (A10)	Cortex	Emotionality
Mesolimbic	VTA (A10)	NAc	Reward and Desire
Tuberoinfundibular	Arcuate Nucleus (A12)	Median Eminence	Regulation of prolactin release
Incertohypothalamic	Zona Incerta (A13) Periventricular region (A14)	Various hypothalamic nuclei, thalamus	Sexual arousal and copulation
Diencephalospinal	Hypothalamus (A11)	Spinal cord	Contraction of penile striated muscles

Central dopaminergic pathways. Within the CNS, three major dopamine pathways exist; the nigrostriatal, mesocortical/mesolimbic and tuberoinfundibular systems which influence motor function, mood, reward and neuropeptide release. There are two additional minor dopamine pathways; the incertohypothalamic and diencephalospinal systems which are believed to modulate elements of sexual behaviour. SN= substantia nigra; VTA=ventral tegmental area; Nac=nucleus accumbens).

incertohypothalamic dopaminergic system located in the zona incerta (A11 and A13 cell groups) and the periventricular region (A14 cell group) (Bjorkland et al, 1975) is thought to be key for the display of masculine sexual behaviour (Edwards and Isaccs, 1991; Maillard and Edwards, 1991). Dopamine exerts its effect via the activation of dopamine receptors of which five (D1-D5) have been identified in the central nervous system (CNS) so far. Each of these integrative dopaminergic systems contribute widely to the regulation of male sexual behaviour ranging from sexual motivation to genital and somatomotor response patterns that define copulation.

The tuberoinfundibular system is believed to have an indirect role on sexual behaviour by influencing prolactin and gonadotrophin secretion which subsequently affects central release of sex steroids including testosterone (Kruger et al, 2005; Paredes and Agmo, 2004 for review). Similarly, the diencephalospinal dopamine pathway would appear to have a role in enhancing penile rigidity due to the dopaminergic innervation of penile striated muscles (Holstege et al, 1996). However, there is insufficient behavioural data to confirm an overall importance of these two dopamine circuits in regulating sexual performance.

The nigrostriatal and mesolimbic dopamine pathways are reportedly important for controlling sexual motivation (Hull et al, 1999 for review; Hull and Dominguez, 2006), as inferred by studies demonstrating that these two systems are critical for sexual appetitive behaviour and have priming properties to allow the animal to respond to incoming sexual stimuli more readily and to enhance sensorimotor output (Dominguez and Hull, 2005; Jones and Robbins, 1992; Salamone, 1992). However, a role for these two systems in facilitating consummatory parameters requires further investigation.

Much interest has surrounded the role of the incertohypothalamic dopamine system (located in the A11 A13 and A14 cell groups), primarily due to its projections to key brain structures (namely the MPOA, PVN and SON) (Bjorkland et al, 1975) which are important for the expression of masculine sexual behaviour. Lesioning of the zona incerta (which contains the dopamine cell bodies of the incertohypothalamic system), had clear inhibitory effects on copulatory behaviour (including penile

erection, intormission and ejaculation) but had no effect on parameters associated with sexual motivation (Edwards and Isaacs, 1991; Maillard and Edwards, 1991). In addition, lesions of the incertohypothalamic system in female rats markedly disrupts the preovulatory surges in LH and prolactin (Sanghera et al, 1991) which suggests that in males, the same effect may also be observed with regard to testosterone's effects on sexual responses. Also, selective ablation of the MPOA and therefore its incertohypothalamic-derived dopamine inputs, results in a profound inhibitory effect on copulatory function as evidenced by a substantial reduction in the expression of mounts, intromissions and ejaculations (Arendash and Gorski, 1983; Paredes and Agmo, 2004 for review, Paredes, 2003). Thus, the incertohypothalamic pathways may have direct (via dopaminergic projections to sexually responsive brain nuclei) and indirect (via testosterone) roles in centrol control of consummatory phases of masculine sexual behaviour where they may mediate the steroidal components associated with the pro-sexual effects of dopamine.

In conclusion, it is very likely that all the major dopamine systems and in particular the nigrotriatal, mesolimbic and incertohypothalamic pathways act in concert to prepare an animal for responding to sexually exciting stimuli and to promote a range of behaviours that define a sexual encounter.

1.8. 2 Dopamine receptors

As previously mentioned, five dopamine receptors exist in the CNS and comprise D1-like (D1 and D5) and D2-like (D2, D3 and D4) receptor subgroups. The receptors can be divided into two separate subgroups depending on the transduction system to which they are coupled to (1) D1-like receptors (D1 and D5) which positively activate adenylate cyclase and D2-like receptors (D2, D3 and D4) which are negatively or not coupled to the enzyme. There is generally widespread expression of all dopamine receptors in the brain with abundant levels of D1 and D2 receptors and moderate expression of D3, D4 and D5 receptors (Ariano et al, 1989; Bitner et al, 2006; Defagot et al, 1997; Khan et al, 1998; Mansour et al 1990; Missale at al, 1998 for review). D1 and D2 receptors are found in the striatum, cortex, hypothalamus, olfactory bulbs and substantia nigra (Levey et al, 1993; Khan 1998). D3 receptor expression is more restricted, with the nucleus accumbens, olfactory tubercles and the

Islands of Calleja showing moderate to high levels of the D3 receptor (Khan et al, 1998). In comparison to D2 receptors, D4 receptor levels appear to be less abundant in subcortical structures. The cortex, hippocampus and striatum have all been shown to possess D4 receptors (Defagot et al, 1997; Khan et al, 1998) (See figure 1.6 for brain maps of dopamine D2, D3 and D4 receptor distribution). Finally, D5 receptor expression in the rat brain is comparatively scarce, however, D5 receptors have been shown to exist in the striatum, cortex, substantia nigra pars compacta and nucleus accumbens (Khan et al, 2000). Since most published work has implicated a role of the D2-like (D2, D3, D4) in mediating the pro-erectile effects of dopamine agonists, this thesis will concentrate on D2, D3 and D4 receptors.

The erectogenic response to systemic and intra-MPOA administration of D1 agonists has been reported before (D'Aquila et al, 2003; Markowski et al, 1994). Similarly, D5 receptors have been implicated in sexual reward (Kudwa et al, 2005). However, it is the D2-like receptors that have the most convincing role in the mediation of penile erection. Table 1.2 illustrates some dopamine D2-like receptor ligands that are known to augment the expression of penile erection in rats. It is apparent that stimulation of D2-like receptors has a profound facilitative effect on penile erection, particularly via D4 receptors. Much attention has been drawn to the role of the D4 receptor during penile erection due to the introduction of highly selective pharmacological tools. Unfortunately, such highly specific dopamine receptor drugs have yet to be developed for the D2 and D3 receptors due to both these receptors demonstrating a high degree of sequence homology. D2 and D3 receptor ligands that are currently available either show the same or only marginal selectivity at D2 and D3 receptors. Thus, results from behavioural studies employing D2 and D3 receptor ligands should be interpreted with caution.

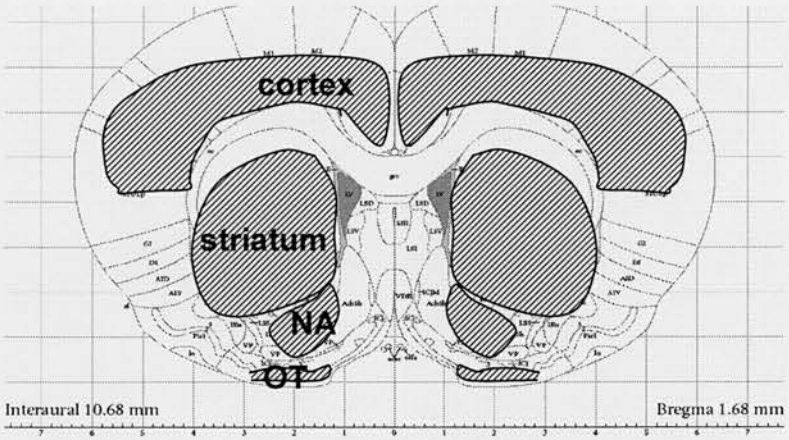
1.8.3 Dopamine and sexual behaviour

Female sexual behaviour

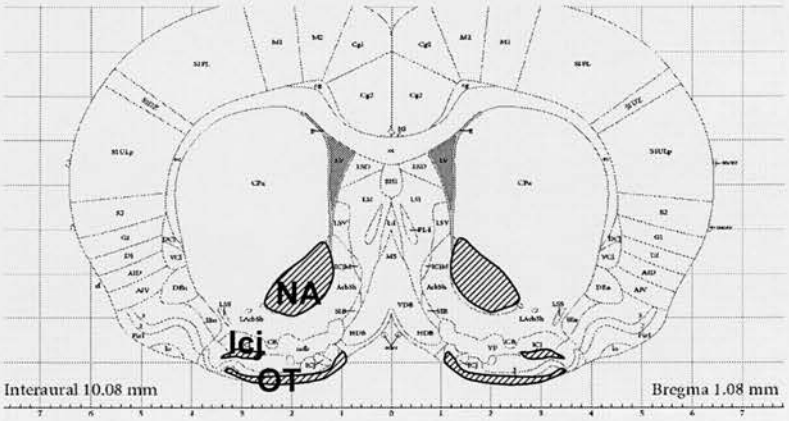
The role of dopamine in female sexual function is more ambiguous than in males. Some findings have shown dopamine to participate in the activation of those pathways controlling sexual motivation and sexual reward (Paredes and Agmo for review, 2004). However, its involvement in female sexual

Figure 1.6: Distribution of D2-like receptors in the rat brain

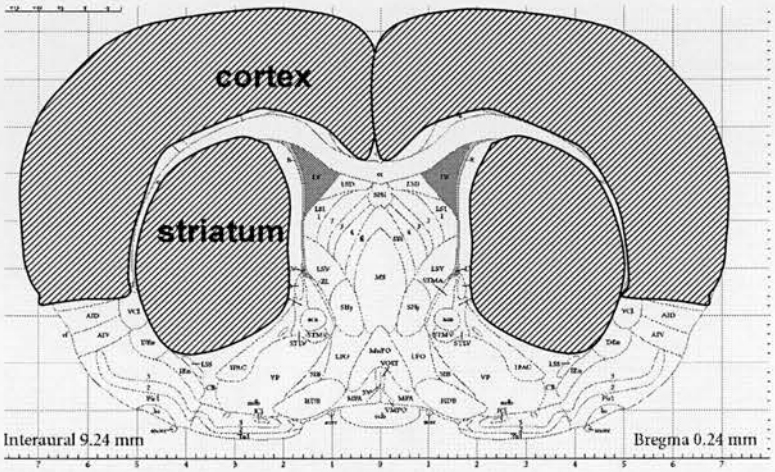
(A) D2 receptors



(B) D3 receptors



(C) D4 receptors



Brain regions in coronal view the rat that show the highest levels of D2 (A), D3 (B), and D4 (D) receptor expression (shaded regions). D2, D3 and D4 receptor expression are not exclusive to these nuclei, however, these brain regions possess comparatively higher levels of each receptor. NA=nucleus accumbens; Icj=islands of calleja; OT=olfactory tubercles.

Table 1.2: The effects of dopaminergic ligands on penile erection

Dopaminergic ligands	**erectile response**	**references**
D2/D3R ligands		
Quinelorane (agonist)	facilitates penile erection, high doses inhibit penile erection	Bitran et al, 1989; Doherty and Wisler, 1994; Eaton et al, 1991; Pomerantz, 1991.
Quinipirole (agonist)	facilitates penile erection	Hsieh et al, 2004; Melis et al, 1987; Zarrindast et al ,1982.
Lisuride (agonist)	increases ICP rises	Chen et al, 1999
L-741,626 (antagonist)	decreases penile erection	Millan et al, 2002
Sulpiride (antagonist)	reduces penile erection; blocks increases in ICP	Chen et al, 1999; Ferrari et al, 1993; Zarrindast et al, 1992;
D3R ligands		
7-OH-DPAT (agonist)	no effect on ICP, facilitates penile erection	Chen et al, 1999; Ferrari and Giuliano,1995;
Nafadotride (antagonist)	unknown, however inhibits ejaculation	Clement et al, 2007; Kitrey et al, 2007; Stafford and Coote, 2006.
S33084 (antagonist)	reduces penile erection	Millan et al, 2000.
D4R ligands		
PD168077 (agonist)	facilitates penile erection	Melis et al, 2005, 2006.
ABT-724 (agonist)	facilitates penile erection	Brioni et al, 2004.
PIP3EA (agonist)	facilitates penile erection	Enguehard-Gueiffier et al, 2006; Melis et al, 2006.
L-745,870 (antagonist)	reduces penile erection	Enguehard-Gueiffier et al, 2006; Melis et al, 2005, 2006; Succu et al, 2007.
A-381393 (antagonist)	reduces penile erection	Nakane et al, 2005.

reflexes is less well defined. Early studies suggested that dopamine may infact have an inhibitory role in females as inferred by studies utilising dopamine agonists such as quinelorane and analysing their effect on lordosis (sexual reflex displayed by a receptive female rat in response to a mounting male placing his front paws on the female's flanks). These authors found that stimulation of the dopaminergic system reduced the expression and duration of lordosis (Eliasson and Meyerson, 1976; Everitt et al, 1975). In contrast in another study, apomorphine and quinelorane were found to have a facilitatory effect on the lordotic response (Foreman and Hall, 1987; Hamburger-Bar and Rigter, 1975). One important factor to consider when investigating dopamine's role in female sexual behaviour, is the hormonal treatments administered to female rats, which can clearly influence the effect of dopamine agonists on sexual behavioural. Differences in the lordotic response to subcutaneously or orally administered dopamine agonists appear to be partly attributed to the levels of oestrogen and progesterone present. Lordosis in oestrogen-primed rats was enhanced by dopamine agonists; however, this effect was inhibited in rats receiving oestrogen and progesterone injections (Foreman and Hall, 1987). Additionally, microinjection of dopamine or apomorphine into the MPOA or ventromdial hypothalamus enhanced sexual receptivity in rats receiving low doses of oestrogen. Such an effect was not observed in rats injected with a high dose of oestrogen. Furthermore, the dopamine antagonist, haloperidol markedly inhibited lordosis in only those females receiving high concentrations of oestrogen (Foreman and Moss, 1979). So, it can be seen that there is some inconsistency in the literature due to the differences in hormonal treatment employed which makes behavioural data difficult to interpret. More recent studies have aimed to further elaborate on dopaminergic control of female sexual function. Microdialysis studies have shown an increase in dopamine levels in the nucleus accumbens in female hamsters and rats during mating (Meisel et al, 1993; Pfaus et al, 1995) which may be due in part to the actions of progesterone (Matuszewich et al 2000). Selective blocking of D1 receptors attenuates neuronal activation in the MPOA and posterodorsal MeA after manual stimulation of the vaginocervical region (Quysner and Blaustein, 2001).

Thus, the involvement of dopamine in mediating components of sexual behaviour in hormonally-primed female rats requires further investigation. However, the findings so far do provide some promising insight into neural pathways mediating rodent sexual behaviour.

Male sexual behaviour

Many neuropeptides and neurotransmitters have been shown to augment masculine sexual behaviour. However, after extensive lesioning, pharmacological and neurotransmitter release studies, dopamine has emerged as one of the most pivotal neuromodulators that can exert differential roles on sexual behaviour through its widespread diffuse projections in the CNS.

The pro-sexual role of dopamine was first identified in the 1970s after male patients suffering from Schizophrenia and Parkinson's disease (caused by abnormalities in dopaminergic neurotransmission) and treated with the dopamine precursor, L-DOPA, displayed increased sexual libido and hypersexuality (Bowers et al, 1971; Jenkins et al, 1970; Mones et al, 1970; Shapiro, 1973). In animal models many pharmacological studies have shown that some parameters of male sexual behaviour (eg. mount latency, frequency of non-contact penile erections, intromission frequency) can be manipulated by the use of dopamine agonists and antagonists (Brioni et al, 2004; Heaton et al, 2000; Hsieh et al, 2004; Paredes and Agmo, 2004 for review). Furthermore, studies involving the measurement of dopamine and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations in dialysates within certain brain regions before and during copulation, have shown there to be a significant increase in both dopamine and DOPAC concentrations in the MPOA and PVN during presentation of a receptive female and copulation (Hull et al, 1993; Hull et al, 1995; Melis et al, 2003). So, it is apparent that the actions of dopamine in key central nuclei may have a substantial influence on appetitive and consummatory functions of sexual behaviour.

Many brain areas that receive dopaminergic innervation have been implicated in sexual behaviour with the MPOA and PVN in particular considered to be principal sites of dopaminergic neurotransmission during sexual behaviour.

1.8.3.1 MPOA

As previously mentioned, there is overwhelming evidence to suggest that the MPOA is the essential site for male sexual behaviour. Dopamine activity within this nucleus is important for copulatory function (Dominguez and Hull, 2005; Everitt, 1990; Hansen et al, 1982; Hull et al, 1999; Paredes and Agmo, 2004 for review). Microinjection of the non-selective dopamine agonist, apomorphine (at doses of 0.5-2μg), strongly facilitates copulatory behaviour in rats as inferred by the increase in mounting and intromitting behaviour and a decrease in the ejaculation latency (Hull et al, 1986). Administration of the D2/D3 preferring agonist Quinelorane, was shown to exert an excitatory role on seminal emission, suggesting a facilitatory effect during ejaculation (Bitran et al, 1989). Similarly, intra-MPOA injection of the D1 agonist dihydroxyphenyl-tetrahydrothienopyridine (THP) shortened the latency to ejaculate and markedly increased the frequency of ejaculations (Markowski et al, 1994). Additionally, dopamine agonists injected into the MPOA of rats observed as inefficient copulators have been shown to partially restore consummatory elements of sexual behaviour (Scaletta and Hull, 1990). There is also some emerging evidence to suggest a role for MPOA dopamine in the motivational phase of sexual behaviour after D1 agonists delivered into the MPOA increased the length of time male rats spent with receptive females (Beck et al, 2002).

Antagonist studies have revealed that blockade of endogenous dopamine actions in the MPOA have revealed marked impairment of male sexual behaviour. The relatively non-selective dopamine receptor antagonists, cis-flupenthixol and haloperidol given systemically or directly into the MPOA, delayed copulation and rats exhibited significant reductions in the frequency of various copulatory parameters including non-contact erections and a reduction in the time spent with a sexually receptive female (Pfaus and Phillips, 1989; Warner et al, 1991), suggesting disruption to sexual desire pathways

Dopamine release studies have shown that during exposure to an inaccessible oestrus female and during copulation, there are significant increases in dopamine levels in the MPOA. Control males either exposed to inaccessible males or permitted free use of the running wheel did not display such an apparent rise in MPOA dopamine release (Hull et al, 1993; Hull et al, 1995). Thus, the elevation in dopamine release appears to be correlated with copulatory-specific behaviours.

Therefore, it seems that dopaminergic neurotransmission in the MPOA is a necessary component for the expression of anticipatory and consummatory sexual parameters which may be mediated via specific neural circuitries residing in the MPOA.

1.8.3.2 PVN

In comparison to other known brain structures that are recognised as having an integral role in erectile responses, the PVN is one of the most sensitive brain areas that is responsive to the pro-erectile effects of the dopamine agonist apomorphine (Chen et al, 1999; Melis et al, 1987). Moreover, lesioning of the PVN abolishes the erectogenic response induced by apomorphine (Argiolas et al, 1987). Such a finding has led some researchers to believe that the PVN is the specific central site where dopamine acts to facilitate penile erection. Intra-PVN and systemic delivery of apomorphine and other dopamine agonists (particularly D4) are profoundly pro-erectile (Brioni et al 2004, Cowart et al, 2004; Hsieh et al, 2004; Melis et al, 2005; Rampin et al, 2001) and can elicit remarkable increases in ICP (Chen et al, 1999). On the other hand, central or microinjection of dopamine receptor antagonists into the PVN clearly disrupts the expression of penile erection in anesthetised and conscious male rats (Chen et al, 1999; Hsieh et al, 2004; Melis et al, 2006).

Pairing of male rats with sexually receptive, yet inaccessible female rats is sufficient to induce a dramatic rise in dopamine concentrations in the PVN, with the highest levels observed during copulation (Melis et al, 2003). Furthermore dopaminergic ligands are known to activate paraventricular neurons as evidenced by a significant increase in Fos expression (Bitner et al, 2006).

Thus, taken together the data suggest that the PVN is a crucial site for mediating penile erection induced by central dopamine pathways.

1.8.3.3 SON

Currently to date there are no data demonstrating a role for dopamine in the SON during sexual behaviour. What we do know is that supraoptic neurons are stimulated during intromission and upon

ejaculation (Caquineau et al, 2006; Pattij et al, 2005). Thus, such preliminary data gives some indication that the SON is another structure where dopamine may potentially act to exert its behavioural effects, those of which may facilitate sexual behaviour.

1.8.3.4 NAc

The role of dopamine in the NAc and the regulation of erectile function is not as well established as other brain regions. Similar to the PVN, appetitive and consummatory elements of male sexual behaviour enhance dopaminergic neurotransmission in the NAc (Pfaus et al, 1990; Wenkstern et al, 1993). Dopamine depleting lesions have only minor effects on sexual behaviour but relatively modest effects on penile erection elicited in response to female oestrus cues (Kippin et al, 2004; Liu et al, 1998). Unlike the MPOA and PVN, injection of apomorphine into the NAc does not have any effect on erectile responses (Melis et al, 1987). Interestingly, such impairments in erectile function are only observed in lesioned naive rats and not sexually experienced rats. Thus, it seems the NAc appears to be of marginal importance during the expression of dopamine-mediated sexual behaviour. Since dopaminergic fibres originating in the PVN project to the NAc (Pinto et al, 2003) it is very possible that dopaminergic activation of NAc during penile erection is secondary to increased activity in the PVN as inferred recently by a study showing that dopaminergic stimulation in the PVN induces an increase in dopamine levels in the NAc during penile erection (Succu et al, 2007).

1.9 Role of androgens in penile erection

In addition to the previously mentioned neuromodulators that can mediate the erectile response; sex steroids play a critical role in the expression of male sexual behaviour. Sex steroids (namely, testosterone and oestrogen) are synthesised in gonadal tissue and the adrenal glands and have a pivotal role in stimulating the development of primary and secondary sex characteristics (Wilson and Davies, 2007 for review). In addition to their role in sexual differentiation during developmental and pubertal phases, these sex steroids also act in the brain and are crucial for the expression of masculine sexual behaviour in many species (Baum, 2003).

In males, testosterone is the main circulating steroid that is metabolised by the enzyme, aromatase to oestrogen (Ryan et al, 1972). Testosterone and oestrogen act via their respective receptors, namely the androgen and oestrogen receptor and are believed to prime neural circuits regulating sexual function. The importance of these neurosteroids was first demonstrated by Stone and Colleagues (1939) who showed that castration completely abolished masculine sexual behaviour in male rats. Since then, the same effect has been observed in various other species after gonadectomy (Baum, 2003). It is generally accepted that androgens are critical for modulating penile erection and maintaining penile rigidity via central and peripheral actions. Preclinical and clinical studies have shown that castration markedly impairs erectile function and hypogonadal men treated with androgen supplements show an improvement in the achievement and maintenance of penile erection (Kupelian et al, 2006; Traish and Guay, 2006). Similarly, in rats who underwent bilateral orchidectomy and displayed impaired penile erection, systemic infusion of increasing doses of testosterone produced erectile events similar to that of intact rats (Armagan et al, 2006). Systemic or central delivery of testosterone or oestrogen has been shown to have strong pro-sexual effects in prepubertal and castrated male rats (Baum, 2003; Bialy and Sachs, 2002; Sachs and Leipheimer, 1988; Manzo et al, 1999). Furthermore, blocking the actions of the converting enzyme, aromatase through the application of antagonists inihibits the pro-copulatory effect of testosterone. In the same study, systemic administration of oestrogen fully restored male sexual behaviour (Clancy et al, 2000; Vagell and McGinnis, 1997). Thus, these neurosteroids are of fundamental importance in regulating copulatory behaviour. However, precisely where the neurosteriods act to exert the effect is not presently clear. Androgen and oestrogen receptors are abundantly expressed in many regions of the CNS including the MPOA, SON, PVN, BNST, MeA and olfactory bulbs (Bingham et al, 2006; Greco et al, 1998; Portillo et al, 2006). Additionally, the MPOA and MeA have both been identified as particularly important sites for mediating the actions of testosterone during sexual arousal and copulation (Putnam et al, 2005; Sato et al, 2005). Thus, it is apparent that the sex steroids act within specific brain structures (presumably the MPOA and MeA) to influence central pathways mediating male sexual behaviour. However, identification of those neurochemical correlates that are sensitive to androgenic effects is still in its infancy.

1.9.1 Pathways mediating the effects of sex steroids

Galanin-containing neurons were shown to be partly mediate the effects of sex steroids after microinjection of galanin into the MPOA facilitated sexual behaviours in testosterone- and oestrogen-primed male and female rats (Bloch et al, 1996; Bloch et al, 1998). The interaction between oestrogen and oxytocin during rodent sexual behaviour in females has been well documented (Agmo et al 2008; Giraldi et al, 2004 for review); however, in male rats very little is known regarding the relationship between sex steroids and oxytocin during the expression of sexual behaviour. In the male reproductive tract, smooth muscle contractility and prostate function are believed to be mediated in part by local circulating oestrogens (Nicholson and Jenkin, 1995; Vignozzi et al, 2004). However, in the one study that examined a possible link between sex steroids and central oxytocin pathways controlling erectile function, they demonstrated that testosterone but not oestrogen restored the erectile response to centrally administered oxytocin in conscious male rats (Melis et al, 1994). This finding is further substantiated by many clinical and pre-clinical studies highlighting dopaminergic targets as one particular group of target cells that are sensitive to circulating levels of testosterone.

1.9.2 Dopamine and testosterone

When measuring erectile responses in castrated rats treated with the classical pro-erectile agonist, apomorphine, rats remained unresponsive until increasing doses of testosterone were administered (Heaton and Varrin, 1994). The dopamine D2/D3 agonist, Quinelorane, acting in the MPOA requires physiological levels of testosterone to induce copulatory behaviour (Charles and McGinnis, 1992). Furthermore, testosterone was shown to influence nitric oxide activity and mating behaviour by increasing dopaminergic neurotransmission in the MPOA (Hull et al, 1997; Putnam et al, 2001, 2005). This androgen-sensitive effect on dopaminergic neurotransmission has also been observed in humans. Males with c-51hronically low levels of plasma testosterone displayed marked erectile dysfunction which could not be restored with apomorphine. Such dopamine-mediating drugs only elicited a pro-erectile response once plasma testosterone levels were increased to physiological levels (Foresta et al,

2004). Thus, these findings add to a growing number of studies suggesting a critical threshold level of circulating androgens must exist to facilitate erectile function

The precise mechanism whereby testosterone acts has yet to be established. However, it is believed that androgens act locally in penile tissue to stimulate the production of nitric oxide and help restrict venous outflow from the pelvic region (Mills and Lewis, 1999). Additionally, androgens are thought to act on central pathways (specifically nitrergic fibres) that regulate penile erection, with the MPOA and PVN known to be particularly sensitive to the effects of androgens. The MPOA appears to be an important androgen-sensitive brain area as sexual behaviour in castrated males is restored only after intra-MPOA delivery of testosterone (Putnam et al, 2001). In addition, ICP responses to electrical stimulation of the MPOA were completely abolished after castration and then restored after testosterone replacement (Suzuki et al, 2007). In the PVN, the erectogenic response to micro-injection of the nitric oxide precursor, L-arginine, is heavily dependent on the presence of testosterone, as evidenced by the lack of erectile response in male castrates lacking testosterone supplements (Wu and Hu, 2002).

In addition to a functional role in the brain, androgens are also believed to act in the lumbosacral spinal cord during reflexive erections (Giuliano and Rampin, 2004). Such a finding was inferred after direct delivery of testosterone to the lumbosacral spinal cord was shown to improve the occurrence of reflexive erections after castration (Hart and Haugen et al, 1968). Subsequent studies have revealed that there is abundant expression of androgen receptors in spinal cord nuclei controlling activity of perineal striated muscles, penile erection and copulation (Jordan, 1997; Watkins and Keast, 1999). Thus, it seems that androgens may exert their effects on penile erection by acting at multiple sites in the peripheral and central nervous system.

The MPOA, PVN and spinal cord contain dopaminergic cell bodies and fibres (Bjorklund et al, 1975; Weil-Fugazza and Godefroy, 1993) and express androgen receptors (Greco et al, 1998; Hart and Haugen, 1968; Jordan, 1997). In addition, all three serve as important integrative sites during sexual behaviour and penile erection (Giuliano and Rampin, 2004). Therefore, it seems entirely plausible

that centrally-acting androgens may act in these three sites to modulate dopaminergic neurotransmission and so influence masculine sexual behaviour. However, the involvement of other androgen-sensitive brain regions such as the medial amygdala (MeA) in erectile function should not be discounted.

1.10 Dopamine-Oxytocin Interactions

When administered centrally in the male rat, dopamine receptor agonists and oxytocin exert a facilitatory effect on almost all aspects of male sexual behaviour, particularly penile erection (Paredes and Agmo, 2004 for review; Bancroft, 2005; Donatucci, 2006; Kita et al, 2006; Hillegaarte et al, 1997). Such findings have led researchers to believe that central dopaminergic and oxytocinergic pathways may interact with each other to mediate erectile function, although it has yet to be established whether such an interaction is synergistic, convergent or sequential. As previously mentioned, the MPOA and the PVN are two hypothalamic brain nuclei that have been shown to be critical integrative sites for male sexual behaviour (Paredes and Agmo, 2004 for review). Additionally, there seems to be an emerging role for the SON (Caquineau et al, 2006; Pattij et al, 2005). The MPOA and PVN have both been shown to be highly responsive to the pro-erectile effects of dopamine receptor agonists and to oxytocin (Melis et al, 2005; 2006; Argiolas and Melis, 2005 for review; Caldwell et al, 1989; Hull et al, 1986, Pehek et al, 1989). Intra-MPOA or –PVN injection of dopamine receptor antagonists strongly inhibit dopamine agonist-induced penile erection (Warner et 1991; Melis et al, 2005). In addition, systemic delivery of the dopamine antagonist, clozapine was shown to inhibit penile erection after central administration of oxytocin (Martino et al, 2005).

Such dopamine-mediated pharmacological effects have yet to be established regarding the SON. Thus, these three brain nuclei make interesting candidates for potential integration sites of central dopamine and oxytocin neurotransmission in the regulation of penile erection.

A variety of technical approaches have been employed to investigate the interaction between central dopamine and oxytocin neurotransmission during penile erection in the male rat. The evidence falls into three primary categories: neuroanatomical, oxytocin release and behavioural pharmacological

studies; some of which are more convincing than others for a direct dopamine-oxytocin link. These will be reviewed in the following sections.

1.10.1 Neuroanatomical evidence

At the cellular level, morphological studies have revealed that terminals of dopaminergic neurons originating in the incertohypothalamic system lie in close apposition to somatic and dendritic processes of the magnocellular and parvocellular oxytocin neurons in the PVN and SON (Buijs et al., 1984; Decavel et al., 1987) which is suggestive of a direct dopaminergic influence. Immunocytochemical studies have revealed abundant expression of D1 (Czyrak et al., 2000) and D4 receptors (Defagot et al., 1997; Bitner et al., 2006) in the PVN and SON (Defagot et al., 1997) of male rats; and additionally, D1 and D5 receptors are expressed in the paraventricular and supraoptic nuclei in primates (Rivkees and Lachowicz, 1997). D2, D3 and D4 receptors were found to be expressed in the SON (Khan et al, 1998), however this has not been investigated in the MPOA or PVN. The neuronal phenotype expressing dopamine receptors remains largely unknown. It is often assumed that dopamine acts on dopamine receptors expressed in oxytocin cell bodies to elicit penile erection, and it has been hypothesised that endogenous dopamine binds to and activates oxytocin cells via D2-like receptors, suggesting a direct action of dopamine on oxytocin cell bodies and dendrites. However, it is important to add that up to now there has been no immunocytochemical confirmation of such a cellular arrangement. Furthermore, it has been harder to prove that endogenous dopamine acts on oxytocin cells *in vivo.*

1.10.2 Oxytocin release studies

One indicator of oxytocin neuron activity is oxytocin release, and several reports have demonstrated that dopamine plays a dynamic role in regulating this. Early *in vitro* studies conducted in the 1970s demonstrated that dopamine and the dopamine receptor antagonist, haloperidol, were able to significantly increase and decrease oxytocin release, respectively, from hypothalamic explants. Moreover, i.c.v. or peripheral administration of dopamine or the non-selective dopaminergic agonist apomorphine increased release of oxytocin into the blood, the hypothalamus, and at other

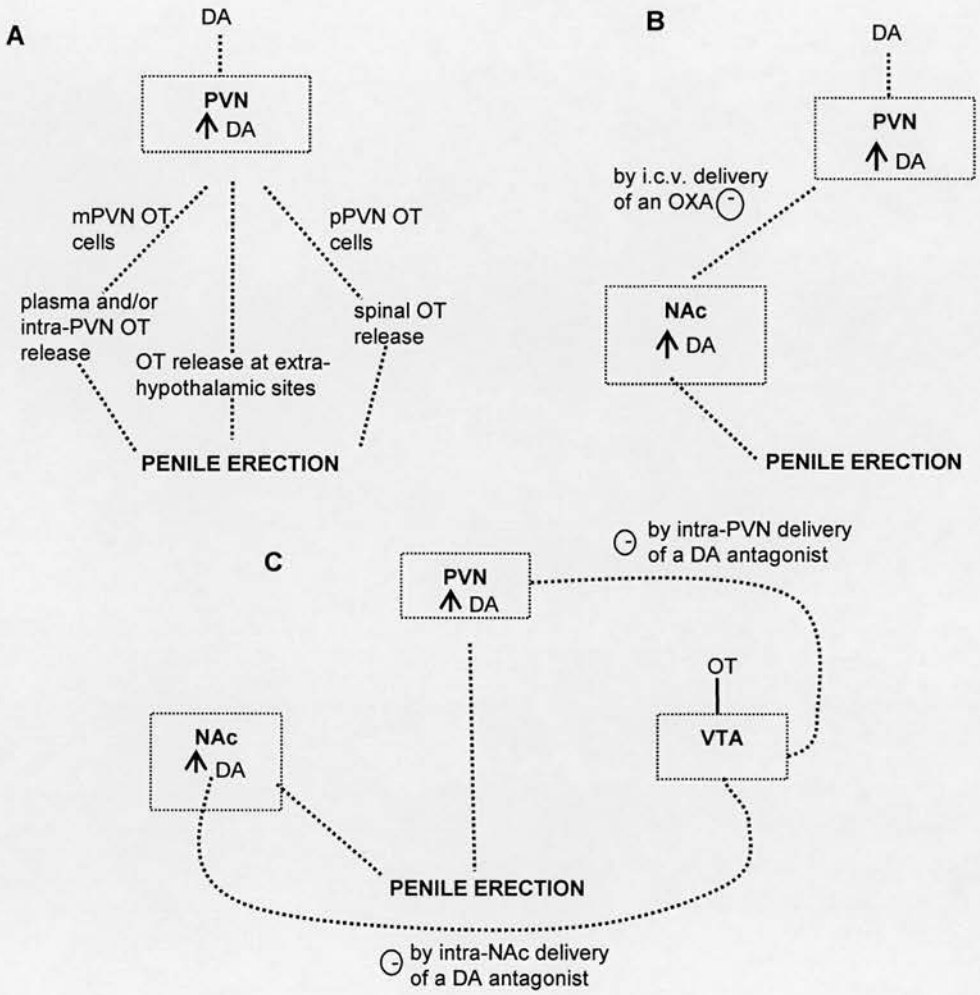
extrahypothalamic sites in various rat and primate models (Bridges et al., 1976; Melis et al., 1990; Cameron et al., 1992; Melis et al., 1992; Argiolas, 1999) (See Figure 1.8A). It is unclear as to which dopamine receptor(s) are involved in central oxytocin release. Electrophysiological studies may shed some light on this: the D2/D3 receptor agonist quinpirole, but not the D1 agonist SKF38393, was able to depolarise oxytocin neurons in the SON *in vitro*. In the same study dopamine-induced depolarisation was antagonised by the D2-like preferring receptor antagonists, sulpiride and spiperone (Yang et al., 1991), implicating the involvement of D2-like receptors in the excitation of supraoptic oxytocin neurons. As previously stated, oxytocin neurons in the hypothalamus are assumed to be innervated by incertohypothalamic-derived dopaminergic fibres, although magnocellular oxytocin cells (in the PVN) are also innervated by intra-PVN dopaminergic fibres (Jourdain et al, 1999). Thus, there could be a predominantly intra-PVN dopaminergic innervation of magnocellular oxytocin neurons and, conversely, an incertohypothalamic innervation of parvocellular oxytocin neurons, which may help to explain the apparent differential involvement of these neurons in sexual behaviour as evidenced by the selective activation of magnocellular oxytocin neurons during intromission and ejaculation (Pattij et al, 2005; Caquineau et al, 2006) and the activation of parvocellular oxytocin neurons during penile erection (Witt and Insel, 1994; Kita et al, 2006).

So, the above evidence indicates that dopamine receptor subtypes are expressed in various hypothalamic nuclei. Although the neuronal phenotype expressing these receptors is unclear, at least some are expected to be oxytocinergic. Such an anatomical arrangement provides support for a potential dopamine-oxytocin link in these key hypothalamic nuclei. It could be postulated that endogenous dopamine may act via D1-like and/or D2-like receptors in the MPN, SON and PVN to activate the hypothalamic oxytocin system. Potentially important sites for the subsequent release of oxytocin include the PVN, hippocampus (Melis et al, 1992) and spinal cord (Giuliano et al, 2001).

1.10.3 Behavioural pharmacological evidence

The proposed inter-dependent relationship between dopamine and oxytocin neurons in the PVN during penile erection was substantiated by the attenuation of apomorphine-induced penile erection after bilateral lesioning of the PVN (Argiolas et al, 1987a) that depletes central oxytocin levels in

Figure 1.8: Dopamine and oxytocin link during PVN-mediated penile erection



Involvement of dopamine (DA) and oxytocin (OT) during PVN control of erectile function. Based on references, penile erection mediated via the PVN is influenced by three pathways investigated thus far. (A) DA action in the PVN stimulates release of OT within the PVN, plasma, spinal cord and extra-hypothalamic sites to elicit penile erection. (B) DA action in the PVN also stimulates DA release in the NAc and penile erection which can both be antagonised by an oxytocin antagonist (OXA). (C) OT action in the VTA also increases DA levels in the NAc and PVN concomitantly to penile erection which can be blocked by intra-NAc and intra-PVN delivery of DA antagonists. In summary, DA stimulation of the PVN induces OT release at central sites which facilitate penile erection perhaps via actions on incertohypothalamic and mesolimbic dopamine neurons. DA: dopamine, OT: oxytocin, VTA: ventral tegmental area, OXA: oxytocin antagonist, ⊖: inhibitory effect.

extra-hypothalamic brain areas (Hawthorn et al., 1985). Stimulation of penile erection by apomorphine or the selective D4 receptor agonist, PD 168077 is also prevented by i.c.v., but not intra-PVN delivery of an oxytocin receptor antagonist (Melis et al, 1992; Melis et al, 2005), suggesting that oxytocin receptors located outwith the PVN are involved in mediating apomorphine-induced penile erection.

Thus, it is not clear as to whether during penile erection, dopamine increases oxytocin levels in the PVN (via magnocellular cells) and/or at sites outwith the PVN (via parvocellular cells) such as in the spinal cord where pro-erectile centres exist (Veronneau-Longueville et al, 1999) or the hippocampus (Melis et al, 1992). There is increasing evidence to suggest that parvocellular oxytocin neurons are part of the neural network controlling penile erection (Witt & Insel, 1994; Veronneau-Longueville et al, 1999; Kita et al, 2006). It is generally believed that apomorphine-induced penile erection involves, at least in part, release of oxytocin at extra-hypothalamic areas via these parvocellular fibres; however, intra-hypothalamic oxytocin release and action cannot be ruled out.

As stated above, dopamine agonist-induced penile erection can be inhibited by a synthetic oxytocin receptor antagonist (Argiolas et al, 1987b); however, oxytocin-induced penile erection is not inhibited by the dopamine receptor antagonist, haloperidol (Melis et al, 1997). This would suggest that dopamine may act upstream to oxytocin in the regulation of penile erection but not vice versa. Interestingly, in another study, penile erection elicited by oxytocin was antagonised by the generally non-selective dopamine receptor antagonist, clozapine (although clozapine does have slightly more affinity for D2-like than D1-like receptors) (Martino et al, 2005) which contradicts the previous finding. So, it is not clear if there is a dopamine-oxytocin or oxytocin-dopamine interaction that results in the stimulation of erectile pathways. Therefore, there may be a synergistic or dual activation of both the dopaminergic and oxytocinergic systems that may have a parallel involvement in the mediation of penile erection. Since haloperidol (D2-preferring) did not block erectile responses to oxytocin and clozapine (D4-preferring) did, it is interesting to postulate that D2 receptors may be located upstream and D4 receptors located downstream from oxytocin cells. Thus, D2 and D4

receptors may differentially regulate penile erection via oxytocinergic and/or non-oxytocinergic pathways.

Many of the current studies support a dopamine-oxytocin relationship in the PVN in relation to erectile function. However, there are some new findings that implicate other brain nuclei and suggest a complex communication network exists between the PVN and other brain nuclei known to be involved in sexual behaviour. Apomorphine and PD168077 (D4 receptor agonist) given intra-PVN have been shown to increase the levels of dopamine and its metabolite, DOPAC, in the NAc, which occurs concomitantly with penile erection, and both the increase in dopamine and penile erection were attenuated by i.c.v. injection of an oxytocin antagonist (Succu et al, 2007). The NAc is generally associated with sexual motivation and sexual arousal; however, a dopamine-oxytocin-dopamine link may exist between the PVN and NAc to elicit penile erection. Interestingly, it has recently been suggested that dopamine and oxytocin neurotransmission form potential underlying neural pathways between the PVN, NAc and the ventral tegmental area (VTA) during penile erection. Paraventricular oxytocin fibres project to the VTA (Roeling et al., 1993) where oxytocin receptors are also located (Freund-Mercier et al., 1987). Intra-VTA administration of oxytocin increased dopamine concentrations in the NAc and PVN concomitantly with penile erection. Both of these effects were attenuated after either intra-VTA injection of an oxytocin antagonist or intra-NAc or intra-PVN injection of the dopamine receptor antagonist, haloperidol (Melis et al, 2007). So, it seems that oxytocin in the VTA may stimulate as yet unknown excitatory pathways that project (perhaps indirectly) back to the PVN to enhance dopaminergic neurotransmission and so facilitate penile erection. Thus, the PVN appears to serve as an important integration site for dopaminerigc and oxytocinergic neurotransmission. Through its complex interactions with other brain regions (particularly the NAc and VTA) (summarised in Figure 1.8), the PVN seems to be a crucial regulatory site for penile erection modulated by dopamine and oxytocin pathways.

From these studies, it can therefore be seen that dopamine-induced pro-erectile effects are not mediated solely by oxytocin pathways originating in the PVN but by other oxytocin-containing nuclei,

such as those in the VTA, that form a much larger and highly integrated network subserving central control of penile erection.

1.11 Dopamine-mediated signalling pathways

It is generally assumed that dopamine receptor stimulation in the PVN triggers a cascade of events that involves the activation of parvocellular oxytocin neurons and the resultant release of oxytocin at extra-hypothalamic sites (Argiolas and Melis, 2004). However, the intracellular signalling pathways and transduction mechanisms activated upon dopamine receptor stimulation and how they facilitate oxytocin release remain to be elucidated. Many studies have shown a wide range of signalling pathways activated by administration of dopamine receptor agonists (all of which cannot be discussed). It is evident however, that many of the signalling pathways are very much dependent on the brain nucleus in which they occur, the cell type involved and cell location as well as the G-protein to which the receptor is coupled to. Therefore it is very difficult to ascribe one particular signalling pathway activated by dopamine in one brain area to another. Evidence for direct and indirect dopamine-mediated signalling pathways and those correlated with penile erection in the MPN, SON and PVN are discussed below.

1.11.1 Potential signalling pathways involving oxytocin cells

It is widely known that D1-like receptors couple positively to adenylate cyclase and are viewed as stimulatory, whilst the D2-like receptors either couple negatively to adenylate cyclase or act via other signalling pathways and are reportedly inhibitory. If the general evidence indicates that D2-like receptors (rather than D1-like) are involved in oxytocin-mediated penile erection, then it is logical to assume such receptor stimulation would inhibit oxytocin release. However, it has been suggested that in the PVN, dopamine-induced oxytocinergic activation may involve a calcium dependent nitric oxide (NO) pathway rather than the classical cAMP pathway (Succu et al, 1998). Intra-PVN injection of ω-conotoxin-GVIA, a selective antagonist of N-type calcium channels inhibits apomorphine- and oxytocin-induced penile erection. Moreover, blockade of the N-type calcium channels attenuates the increase in nitrite and nitrate concentrations (indicators of NO activity) during penile erection (Succu et al, 1998). The NO generator, neuronal nitric oxide synthase (NOS) is abundantly expressed in

oxytocinergic neurons (Ferrini et al, 2001; Xiao et al, 2005) where as a gas, NO can act in a paracrine and an autocrine fashion. Centrally administered NOS inhibitors were shown to prevent dopamine agonist and oxytocin-induced penile erection. Furthermore, the concentration of NO in PVN dialysates increases after administration of apomorphine and D2-like agonists, an effect which occurs concomitantly with penile erection (see Melis and Argiolas, 2005 for review). It has been suggested that dopamine receptor stimulation initiates the opening of the N-type calcium channels in a similar manner to NMDA receptors ie. via coupling to a G protein; however, activation of the N-type calcium channels may be via an as yet unknown intracellular signalling pathway. In line with the former hypothesis, such increased intracellular calcium levels would activate NOS which in turn stimulates NO production and the subsequent activation of oxytocin neurons. However, it is important to add that as yet there is no published data showing that NO directly activates hypothalamic oxytocin neurons. Interestingly, NO has been shown to inhibit the firing pattern of magnocellular oxytocin neurons in the SON (Srisawat et al, 2000) so it is unclear as to how NO donors stimulate penile erection. NO may act intracellulary in oxytocin neurons, secondary to a rise in intracellular calcium levels as previously described. Alternatively, NO may act extracellulary by acting on neuromodulatory inputs to oxytocin cells in the hypothalamus, where local NO production levels can alter excitatory and inhibitory functions (Calka, 2006 for review). Intra-hippocampal delivery of a low dose of NO donors decreased GABA release in dialysates (Getting et al, 1996). Similarly, administration of a high dose of NO-stimulating agents into the hippocampus, enhances glutamate release (Segieth et al, 1995; Sequeira et al, 1997). Thus, NO may stimulate oxytocinergic neurotransmission and penile erection by altering GABAergic and glutamatergic influences in the hypothalamus.

In addition to a NO pathway, dopamine may exert its stimulatory effects in a more direct fashion via an increase in intracellular calcium levels. It has been shown that D2-like receptors coupled to the chimeric G-protein, $G_{\alpha q}$ in transfected cell lines increased intracellular calcium levels upon dopamine stimulation (Moreland et al, 2004); however, it is not known if this G-protein is found in oxytocin cells or whether D2-like receptors would naturally couple to this G-protein physiologically. In the neurohypophysis, activation of dopamine D4 receptors leads to inhibition of potassium currents

(Wilke et al., 1998); thus, D2-like receptors could also have a partial inhibitory effect on cell hyperpolarisation thereby enhancing oxytocin release.

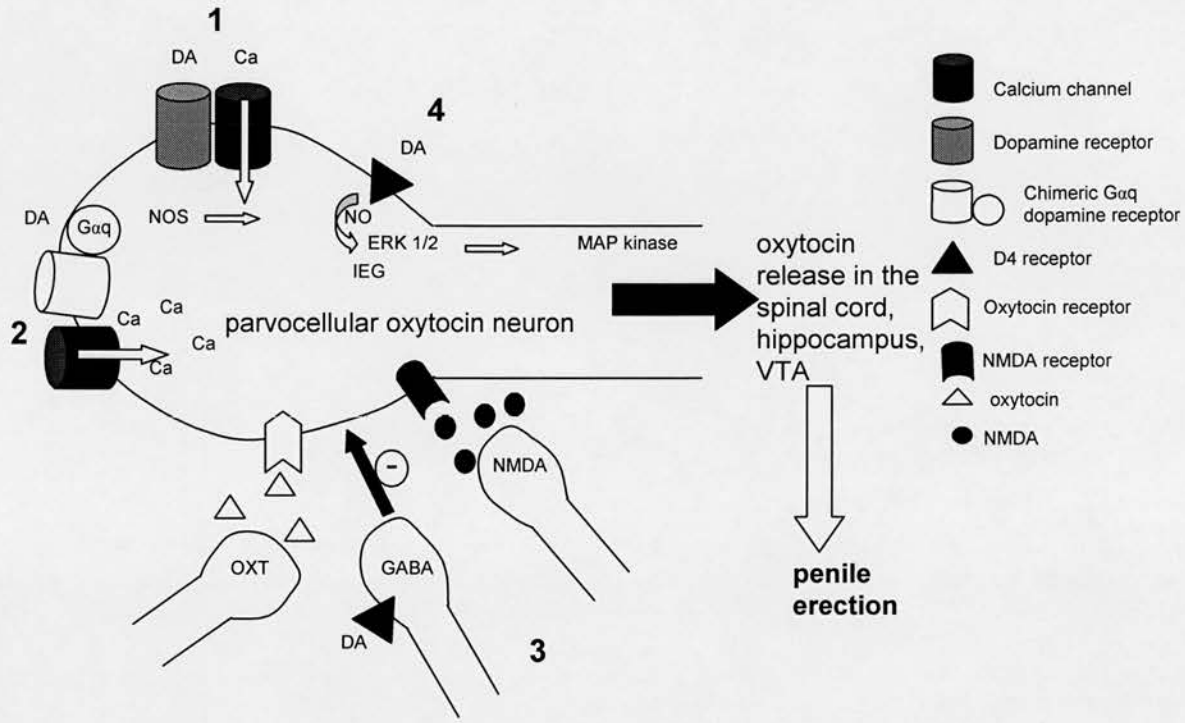
The D4 receptor agonist, PD-168077 has been shown to induce Fos expression and ERK 1/2 phosphorylation in the PVN of male rats (Bitner et al, 2006). Such an effect was antagonised by A-381393, a dopamine D4 receptor antagonist, and SL327, an ERK 1/2 phosphorylating inhibitor. Additionally, D4 receptor expression was co-localised with Fos in PVN neurons, although it is not known if these were oxytocinergic. Unfortunately in this study the gene expression was not correlated with penile erection; however, it does suggest that D4 receptors in the PVN mediate the actions of PD-168077 by activating immediate early gene and/or MAP kinase pathways (Bitner et al, 2006; potential dopaminergic actions on OXT neurons in the PVN are summarised in Figure 1.9).

There does not appear to be any literature reporting a dopamine-oxytocin link in the MPN in relation to sexual behaviour, thus even less is known regarding the potential signalling pathways underlying dopamine-oxytocin interactions in this nucleus. However, it is believed that NO facilitates dopamine release in the MPOA as shown by a comprehensive study carried out by Sato and Hull (2006) where they postulated that activation of a NO-cGMP pathway enhanced MPOA dopamine release. An analogue of cGMP and an inhibitor of its precursor, guanylate cyclase increased and decreased MPOA dopamine release, respectively. In addition, cGMP was shown to concomitantly increase dopamine release and ejaculation frequency. Conversely, inhibition of guanylate cyclase decreased ejaculation frequency (Sato and Hull, 2006). This provides strong evidence for a NO-dopamine link in the MPOA during male sexual behavior; however, as before the neuronal phenotype expressing NOS is not known. Because NO can act as an intra- and intercellular messenger and is present in hypothalamic oxytocin neurons, it could be tempting to suggest an oxytocin-NO-dopamine pathway in the MPN of the MPOA.

1.11.2 Signalling via interneurons

It is known that a complex cellular hierarchy exists in the SON and PVN whereby oxytocin neurons are heavily modulated by inhibitory GABAergic and excitatory glutamatergic neurons, which

Figure 1.9: Potential dopaminergic transduction systems subserving penile erection



Summary diagram of dopamine-mediated signalling pathways during penile erection. Dopamine may act via (1) a calcium dependent nitric oxide pathway (2) the chimeric G-protein Gαq to increase intracellular calcium levels (3) presynaptic inhibition of GABA neurons via D4 receptors, thus disinhibiting oxytocin neurons and leaving them free to respond to excitatory neuromediators (4) D4 receptors to stimulate ERK 1/2 phosphorylation and/or IEG expression to activate MAP kinase pathways, to excite parvocellular oxytocinergic cells and stimulate release of oxytocin in areas such as the spinal cord, hippocampus and VTA. DA: dopamine, GABA: gamma-aminobutyric acid, OXT: oxytocin, NMDA: N-methyle-D-aspartic acid, Ca: calcium, NOS: nitric oxide synthase, NO: nitric oxide, IEG: immediate early gene, ERK: extracellular signal-regulated kinases, MAP: mitogen-activated protein kinase, VTA: ventral tegmental area.

themselves can be influenced by a range of synaptic inputs and neurotransmitters present in the extracellular fluid. GABAergic and glutamatergic neurons heavily synapse with magnocellular oxytocin soma and dendrites in the PVN and SON whereas dopaminergic innervation of these nuclei is comparatively scarce (Jourdain et al, 1999). Thus, it seems reasonable to assume that endogenous dopamine could exert its' effect on oxytocin release by acting indirectly via glutamatergic and/or GABAergic neurons rather than oxytocin neurons directly. Evidence for an intermediary role for GABA includes studies showing that intra-PVN injection of muscimol, the $GABA_A$ receptor agonist, inhibits apomorphine- and oxytocin-induced penile erections and, in parallel, attenuates the increase in nitrite and nitrate concentrations in the PVN (Melis and Argiolas, 2002). In particular, D4 receptor stimulation has been implicated in the disinhibition of GABAergic and inhibition of glutamatergic neurotransmission in the SON by acting presynaptically (Price and Pittman, 2001; Azdad et al, 2003). Although it is unclear as to which system would predominate under physiological conditions, it could be speculated that dopamine-induced inhibition of GABAergic and glutamatergic neurons would somehow increase the responsiveness of the postsynaptic cell, conferring on it a greater sensitivity, free to respond to other excitatory factors such as oxytocin. Thus, it could be that during the initiation of penile erection, dopamine acts presynaptically to disinhibit oxytocin neurons and so facilitate the activation of the hypothalamic oxytocinergic system. One study which contradicts this GABA disinhibition theory showed that Zolpidem, a selective receptor agonist for the $GABA_A$ receptor α subunit, significantly induced Fos expression in magnocellular oxytocin neurons in the PVN; although, this was not correlated with penile erection (Kiss et al, 2006). As previously described, activation of $GABA_A$ receptors on oxytocin neurons is normally associated with increased GABAergic inhibition and, thus, reduced activity in oxytocin neurons. But this study found, contrary to expectations that Fos indicates neuronal excitation; increased magnocellular oxytocinergic activation in the PVN. However, the SON which is predominantly magnocellular did not show such an excitatory effect on neuronal activation. Zolpidem also increased Fos expression in the parvocellular PVN but again the neuronal phenotype expressing the Fos was unknown (Kiss et al., 2006). Taken together, it seems GABA may differentially modulate oxytocin neurons depending on the nucleus they are located in. It is possible that in this study, Zolpidem was interacting with the α subunit in $GABA_A$ receptors located pre- and postsynaptically. This may partly explain the increased

Fos expression in oxytocin neurons. Additionally, $GABA_A$ receptors are comprised of a variety of subunits, each with their own isoforms, thus giving rise to a diverse range of functional $GABA_A$ receptors. It has yet to be demonstrated, but $GABA_A$ receptors activated by Zolpidem in this study may perform in a contradictory manner to conventional $GABA_A$ receptors thereby inhibiting GABAergic neurotransmission (See Figure 1.9).

Thus, there are a range of potential direct and indirect signalling pathways activated upon dopamine receptor stimulation in the hypothalamus. However, further studies are required to elaborate on the activation of behaviourally-specific transduction mechanisms and their correlation with the expression of penile erection.

1.11.3 Dopamine and oxytocin interaction summary

The central neural networks and intracellular signalling pathways controlling penile erection are far from being fully established. However, central dopamine and oxytocin pathways are clearly involved. Evidence suggests that dopamine acts at least partly via the oxytocinergic system and the MPN, SON and PVN are three brain regions that are the most likely sites for dopaminergic control of oxytocin neurons. However, it is still to be established whether endogenous dopamine acts directly and/or indirectly via oxytocin neurons to mediate sexual behaviour: other extrahypothalamic areas mediated by these and other neuromodulators are almost certainly involved. Cross-talk between central dopaminergic and oxytocinergic neurotransmission in the mediation of penile erection appears to be an exciting and intriguing prospect which warrants further investigation. Due to the high incidence and severity of side effects associated with some erectile dysfunction drugs that modulate dopaminergic transmission, it would be interesting to further investigate how central dopamine and oxytocin pathways interact. Such dual participation of dopamine and oxytocin in the control of penile erection could be clinically exploited; particularly the oxytocinergic system, which is a key therapeutic target due to its pro-erectile effects and seeming lack of ability to cause adverse side effects. However, more thorough investigation has to be performed to help establish those circuits that constitute the neuronal basis of male sexual behaviour.

1.12 Thesis aims

So although it is very apparent that central dopamine and oxytocin are clearly pro-erectile, the interactions between these two neuromodulators in the CNS remain poorly understood. Pharmacological, neuroanatomical and oxytocin release studies have all shown support for a relationship between dopamine and oxytocin in the brain and spinal cord during the expression of penile erection. However, specific central sites which integrate dopaminergic and oxytocinergic neurotransmission and by what mechanism are far from being elucidated. Although administering dopaminergic agents into the hypothalamus can alter oxtyocin release in the CNS and plasma, it is not clear where the resultant oxytocin response occurs or if it is due to a direct action by dopamine. Furthermore, if dopaminergic inputs to hypothalamic oxytocin cells can influence the neuronal activity of oxytocin cells, which dopamine receptors mediate the effects of these ligands and where are they located?

As previously mentioned the MPN of the MPOA, SON and PVN are three key hypothalamic nuclei that contain oxytocin neurons and have been implicated in mediating various aspects of masculine sexual behaviour. Furthermore, the MPN and PVN are highly responsive to the pro-erectile effects of dopaminergic ligands. Taken together, current findings have provided some evidence that these brain nuclei may be key anatomical targets where an interaction between dopamine and oxytocin could occur to facilitate penile erection. However, whether dopamine has any effect on these hypothalamic oxytocin neurons during penile erection and knowledge of potential oxytocin release sites is not known. Additionally, oxytocinergic expression of dopamine receptors in the MPN, SON and PVN, which would be indicative of a direct dopaminergic influence on oxytocin neuronal activity, has not been reported in any of these brain regions.

1.12.1 Hypothesis

In order to clarify the potential role of dopamine in the excitation of hypothalamic oxytocin neuronal populations and on oxytocin release in the context of penile erection, we developed the following hypothesis;

Dopamine acts via D2-like receptors located on oxytocin neurons in the MPN, SON and PVN, to stimulate central oxytocin neuron activity and oxytocin release in the spinal cord and facilitate penile erection in the rat.

This thesis has attempted to address the above hypothesis by conducting three main experiments;

(1) In order to investigate the effect of dopaminergic ligands on hypothalamic oxytocin neurons during penile erection and using Fos as a marker of neuronal activation, D2-like dopaminergic ligands were administered centrally to conscious animals and their effect on erectile responses and oxytocinergic activation were analysed.

(2) To investigate whether the lumbosacral spinal cord is one potential oxytocin release site after dopaminergic stimulation, the effect of oxytocin receptor blockade in the lumbosacral spinal cord on dopamine agonist-induced penile erection in anaesthetised rats was examined. Additionally, the ability of a dopaminergic agonist to elicit an increase in oxytocin release in the lumbosacral spinal cord was also investigated.

(3) To provide anatomical evidence for a direct dopamine-oxytocin link in the hypothalamus, the expression of D2-like receptors by oxyocin neurons in the MPN, SON and PVN was examined.

Chapter 2

General Material and Methods

Chapter 2

General Materials and Methods

2.1 Animals

Male Sprague Dawley rats, weighing 225-250g, were used (Charles River, UK) in all experiments. They were housed in groups of 6 (unless otherwise mentioned) and maintained under standard conditions with food and water available *ad libitum*. Rats were maintained throughout on a 12:12h light:dark cycle (lights on from 7am to 7pm); except in the copulation study, rats were placed under reverse light cycle on a 12:12h dark:light cycle (lights on 7pm to 7am). All procedures were approved by the UK Home Office under the Animals (Scientific Procedures) Act 1986.

2.2 Anaesthesia and analgesia

2.2.1 Recovery – gaseous anaesthetic

Prior to any surgical procedures being performed (for male and female rats in the behavioural study), rats were placed in an anaesthetic box containing isoflurane. Once sufficiently anaesthetised, the rat was placed in the stereotaxic frame and maintained by continuous delivery of isoflurane (3% in 600ml/min of oxygen). Isoflurane is a reversible gaseous anaesthetic and muscle relaxant. It acts by potentiating neurotransmission at $GABA_A$ and glycine receptors by interacting directly with proteins and/or indirectly via the lipid bilayer (Tanner et al, 2000).

2.2.2 Non-recovery – liquid anaesthetic

Rats used in the intracavernous pressure and push-pull perfusion studies were terminally anaesthetised with an injection of urethane (1.2g/kg, Sigma, UK). Urethane is often the chosen anaesthetic for this particular study (Allard et al, 2002; Giuliano et al, 2001; Giuliano et al, 2002) because it is less quickly metabolised than sodium pentobarbitone, and rats remain deeply anaesthetised for several hours after a bolus injection. Moreover, urethane has minimal effects on respiration whilst

maintaining spinal reflexes. Urethane acts to enhance function at glycine, $GABA_A$ and nicotinic acetylecholine receptors (Hara and Harris, 2002). In addition, it reduces activity at AMPA and NMDA receptors, thus it seems both inhibitory and excitatory pathways are selectively modified by urethane (Hara et al, 2002).

2.2.3 Analgesia

Once anaesthetised and prior to commencing surgery, rats were injected with an analgesic (Rimadyl, UK, 50µl, s.c.). Rimadyl is a non-steroidal anti-inflammatory (NSAID) agent which acts to relieve pain and reduce inflammation. As with other NSAID's, Rimadyl can partly modulate the humoral and cellular immune response. It acts by inhibiting the enzyme, cyclooxygenase which under normal physiological conditions stimulates prostaglandin release and contributes to the immune response.

2.3 Surgical procedures

On the day of surgery, all surgical instruments including cotton wool, suture thread and needles were autoclaved and instruments and cannulae were dipped in 70% ethanol followed by saline prior to use. Incision sites were previously wiped with iodine and a few drops of 70% ethanol to promote sterility. The lack of the toe-pinch reflex and whisker movement were used as indicators that the rat was sufficiently anaesthetised. After all surgeries, rats were given saline (1ml, i.p.) to help restore any fluid loss and then placed in a heated (37°C) recovery box before returning to their home cage.

2.3.1. Subcutaneous (s.c.) injections

For copulation experiments sexually receptive Sprague Dawley female stimulus rats (200-250g) were used. Ovariectomised females were injected subcutaneously with 15µg/150µl and 1000µg/100µl of oestradiol benzoate and progesterone (both Sigma, UK) dissolved in 100% ethanol and diluted 90% in sesame oil, 4 h and 48 h respectively, before experiment day to induce receptivity.

2.3.2 Intraperitoneal (i.p.) injections

Prior to transcardial perfusion, rats were anaesthetised with an overdose of sodium pentobarbitone (60mg/ml, 0.8ml, Sagatal, Rhone Merieux, Harlow, UK). Care was taken to ensure the injection needle entered the abdominal cavity but avoided puncturing of major organs. Sodium pentobarbitone is a short-acting barbiturate which acts by mimicking the actions of GABA and acting predominantly at postsynaptic $GABA_A$ receptors. It acts to depress and enhance the activity of cationic and chloride channels respectively and so decreases neuronal firing (Belelli at al, 1999: Richards, 2002 for review).

2.3.3 Intravenous (i.v.) injections

In the experiments involving urethane-anaesthetised rats, either urethane (20µl) or apomorphine (250µl) was injected via a previously implanted femoral vein cannula. Urethane or apomorphine were administered i.v. using a 1ml syringe to maintain anaesthesia (and sacrifice animal) and to induce transient increases in intracavernous pressure, respectively.

2.3.4 Intracerebroventricular (i.c.v.) stereotaxic placement of guide cannula

Isoflurane-anaesthetised rats were placed carefully in the stereotaxic frame (ASI Instruments Inc. Michigan, USA); care was taken to ensure the delicate ear bones were not damaged. Lack of any lateral movement whilst secure in the ear bars signified that the head of the rat was in the correct position. A small incision was made in the skin and cranial membranes were cleared from the skull. Lambda and bregma were measured to ensure the skull was level before starting surgery. Prior to implantation of the cannula, a drill was used to make three holes in the skull; two for screws (Plastics One, Vancouver, Canada) and one for the guide cannula (22-guage, Plastics One, Vancouver, Canada). One screw was placed diagonally anterior and another posterior to the cannula implantation site. These two screws would later serve as anchorage for the implanted cannula. To enable direct injection of drugs into the lateral cerebral ventricle, a stainless-steel guide cannula was implanted into a lateral cerebral ventricle (stereotaxic coordinates, 1.6mm lateral and 0.6mm caudal from bregma, inserted to a depth of 4mm from the skull surface). Once the cannula was in place, dental cement

(comprised of dental powder mixed with acetylamide, Simplex Rapid, Kendent Works, UK) was carefully poured into the skin opening around the cannula, making sure the screws, the lower part of the cannula and edges of skin were submerged. Once the dental cement had hardened, the frame clamp was released from the cannula and a snugly fitting protective cap was placed in the cannula.

2.3.5 Ovariectomy (OVX) preparation

Three weeks prior to experiment day, female rats were anaesthetised (as described above) and placed on their ventral surface. Bilateral incisions (approximately 1cm in length) were made in the skin mid-to-lower back, on either side of the spinal cord and just above the gluteus maximus. Careful cuts were made through the layers of fascia and connective tissue to reveal the abdominal wall. A clean vertical cut was made in the abdominal cavity and small forceps were used to locate fatty tissue. The fatty tissue connected to the uterus, ovaries and fallopian tubes was then gently pulled through the incision site, with the ovaries (dark red in colour) clearly distinguishable from the fatty tissue and uterus. Using fine sutures (metric 1.5 (4.0), Surgisilk, Sutures Ltd., UK) a double knot was tied around each fallopian tube and using fine scissors, the ovary and all follicles were removed. The uterus was then carefully placed back in the abdominal cavity and the abdominal wall was stitched using sutures to close the wound. Wound clips (Michel suture clips, 7.5x 75mm, Fine Science Tools, Germany) were then used to close the skin. The wound clips were kept in place for approximately 7-10 days, after which they were removed under light anaesthesia. Prior to and on experiment day, the female rats were primed with s.c. injections of progesterone (Sigma, UK) and oestrogen (Sigma, UK) (as described previously) to induce sexual receptivity.

2.3.6 Intra-PVN injection

The anaesthetised rat was secured in the ear bars of the stereotaxic frame. An incision was made in the skin and the skull (including bregma) exposed. Measures were taken as described previously to ensure the skull was level. For PVN injections, the coordinates used were 1.8mm caudal, 0.5mm lateral (from bregma) and 8.6mm ventral from the skull surface. A 23G hypodermic needle, held in a micromanipulator was lowered vertically to mark the injection site coordinates on the surface of the parietal bone. At this point, a small hole approximately 1-1.5mm in diameter was drilled. The needle

was then lowered vertically into the brain. Using a 10µl Hamilton syringe (Hamilton, Switzerland), drugs were injected in a volume of 2µl over 2 min. After each injection the needle was kept in place for a further 30 sec to allow diffusion of the compound and to prevent backflow into the needle tract.

2.3.7 Intraspinal injection

Intraspinal injection was performed using a binocular microscope (Wild Heerbrugg MS-C, Switzerlan) at 10x magnification. The anaesthetised rat was placed on its ventral surface and an incision was made at the level of the thoracic vertebra which proceeded caudally approx 6-7cm. The junction that lies between the T13 and L1 vertebra level was located by identifying the beginning of the fused sacral spinal vertebrae and then counting rostrally (six vertebra in total) toward the thoracic spinal cord level. An incision was made at the T13 and L1 junction and muscle and connective tissue were carefully removed to expose the dorsal vertebral lamina. Bone surrounding the joint was carefully removed to make a small "window" which exposed part of the lumbosacral (L4-L6) spinal cord. Care was taken to avoid rupturing the central vein or membranes surrounding the spinal cord. The animal was then placed on the flat platform of the stereotaxic frame in a supine position, the upper body elevated and rolled forward towards the tail to expose the spinal cord. Using a micromanipulator, spinal cord injection was performed lateral to the central vein, approximately 1mm deep in to spinal cord tissue. Using a 10µl Hamilton syringe, the injection needle was lowered vertically and drugs were injected in a volume of 3µl over 3 min.

2.3.8 Cannulation of the femoral artery and vein

After preparing the spinal cord and prior to intraspinal injection, anaesthetised male rats were placed in the supine position on a homeothermic blanket (Harvard Apparatus, UK) to maintain body temperature at 37°C. An incision was made approximately 1.5-1.8cm above the penile opening and proceeded laterally towards the knee joint, an opening in the subcutaneous tissue was made and all connective tissue was removed to expose the femoral vein and artery. The femoral vein was

catheterised (0.63mm in diameter, 30cm in length, Portex Ltd., UK) to allow additional doses intravenously (i.v.) as necessary to maintain anaesthesia during the experiment. Additionally, the femoral artery was also catheterised (0.80mm in diameter, 30cm in length, Portex Ltd., UK) for measurement of systemic blood pressure. The same procedure was used to cannulate both the vein and the artery. A ligature (metric 1.5 (4/0), Surgisilk Ltd., UK) was tied in a double knot at the distal end of the vein and another ligature tied loosely in a single knot was placed at the proximal end of the vein. Fine forceps were placed under the vein and opened to stretch the vessel. A small incision was made in the vein to allow insertion of the cannula. Before careful insertion of cannulae into the vein and artery, each catheter was filled with urethane and heparinised saline (25UI/ml, CP Pharmaceuticals, UK) respectively, and all air bubbles expunged. Using thin forceps, approximately 1-1.5cm of the tip of the cannula was carefully inserted into vein. Once the cannula had been inserted into the vessel, the loosely tied ligature at the proximal end was tightened and tied in a double knot once the cannulae were in place. The proximal ligatures of the artery and vein cannulae were finally tied together so that both cannulae lay in the same plane and were secured.

2.3.9 Measurement of corpus cavernosum pressure

After cannulation of the femoral artery and vein, the penis was dissected and a needle inserted in one corpus cavernosum to record intracavernous pressure (ICP). The penile opening was cut and all connective tissue at the base of the penis was carefully removed. Holding the glans penis, the middle third of the penis was denuded of skin and the surrounding connective tissue removed by careful rubbing with a cotton bud. A catheter attached to a 25-gauge needle (25G, 15mm in diameter, 30cm, Venofix, Braun) was stretched to 30% more than its original length and was filled with heparinised saline (25UI/ml). The 25G needle was inserted into one corpus cavernosum in the middle third of the penis for measurement of ICP. The ICP catheter was connected to pressure transducers (EM 750, Eclomatic, Glasgow, UK). Once the needle was in place, the penis was flushed with 100µl of heparinised saline (25U/ml) to ensure there were no holes or leakages in the penile tissue.

In all the intracavernous pressure experiments, catheters were flushed with 100µl saline (Fresenius Kabi, UK) before and after drug injection to wash out urethane and drug in the catheter, respectively. Basal conditions were recorded for 10 min prior to any injection and for 30 min after the injection of apomorphine.

2.4 Procedures for i.c.v. injection via a guide cannula

The animals were housed individually after surgery and allowed 2 days to recover. The conscious rats were handled daily to familiarise them with the injection procedure. On experiment day, each rat was gently restrained in his home cage. The injection cannula was connected by polyethylene tubing filled with vehicle or drug, to a gas-tight 10µl Hamilton syringe that was then inserted into the guide cannula. Drug or vehicle was injected into the left lateral ventricle in 2µl over 2 min via an injection cannula (28 gauge) which extended into the ventricle 1.0mm below the tip of the guide cannula. The animals were allowed to freely move around during drug administration to reduce any stress endured. The injection cannula was kept in place for a further 30 seconds to allow the drug to diffuse from the injection site and to avoid a backflow of the drug into the cannula.

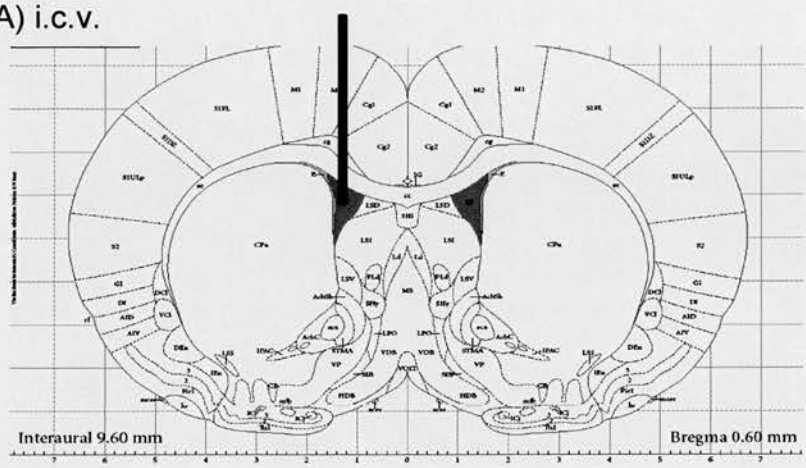
2.5 Examination of injection site

2.5.1 i.c.v.

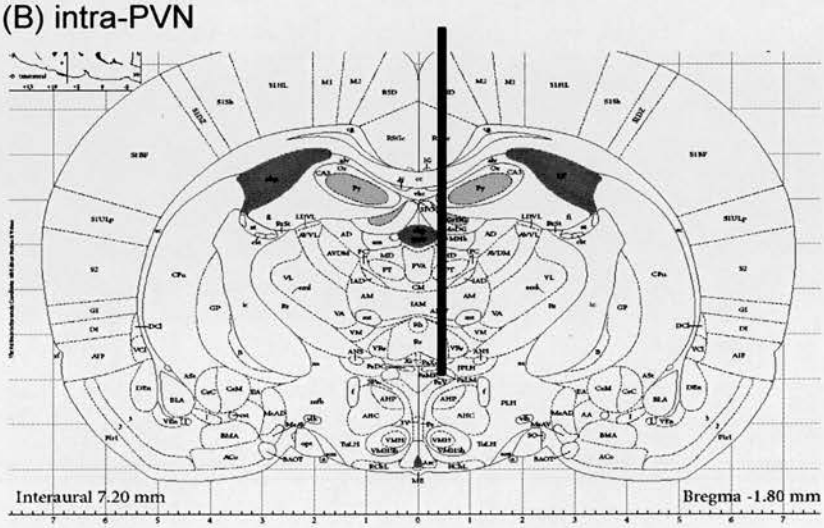
Whilst cutting the brains on the microtome, the site of the ICV cannula tip was localised following the cannula track through a series of brain sections (48µm in thickness). Only animals in which the cannula tip was positioned in the left lateral ventricle were considered for statistical analysis (see Figure 2.1A)

Figure 2.1: Brain maps showing central injection sites

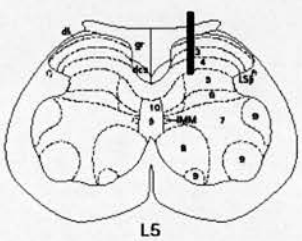
(A) i.c.v.



(B) intra-PVN



(C) intraspinal



Diagrammatic representation of injections sites (filled bars) in the lateral ventricle (A), PVN (B) and L5 spinal level of the lumbosacral spinal cord (C), in coronal view.

2.5.2 Intraspinal and intra-PVN

Once each rat was sacrificed, the brain or L4-L6 region of the spinal cord were then dissected out and stored in formalin (with 4% paraformaldehyde) for 24 hours before being cut on a cryostat (Leica CM 3050, UK) (30μm in thickness). Brain sections containing the PVN or L4-L6 spinal cord sections were mounted onto gelatinised microscope slides (76x26mm, Menzel-Glaser, Braunschweig) (3g gelatine (Sigma, UK) in 600ml dH_2O, once dissolved add 0.3g chromium potassium sulphate (Sigma, UK). Sections were then firstly placed in decreasing ethanol concentrations (100 x2, 90, 70. 50x2 %) followed by methylene blue (Pro-lab diagnostics, UK) and then dehydrated in increasing ethanol concentrations (50x2, 70, 90, 100x2) and histoclear (National Diagnostics, UK) x2 for 2 min each. Sections were then coverslipped and allowed to dry before locating the site of injection using a light microscope (x20 magnification) (See Figure 2.1B and 2.1C).

2.6 Brain and spinal cord tissue processing

2.6.1 Perfusion

Rats were terminally anaesthetised with an overdose of sodium pentobarbitone (60mg/ml, 0.8ml i.p., Sagatal, Rhone Merieux, UK). The toe pinch reflex test was conducted to ensure the rat was terminally anaesthetised. Once the animal had stopped breathing and there was no longer a reflex response, a vertical incision was made in the abdominal cavity which proceeded rostrally toward the thoracic cavity. Using thick forceps to grip the sternum, two cuts were made on either side of the ribcage which was then bent backwards and held open by a clamp to expose the still beating heart. Care was taken to cut the diaphragm and any connective tissue surrounding the heart. A blunt 25G needle attached to a perfusion pump was then inserted into the apex of the left ventricle of the heart and guided towards the aorta. One atria was then snipped immediately afterwards to allow blood to be flushed out of the heart.

Rats were perfused with heparinised saline (200-250ml) until the blood ran clear and then 4% paraformaldehyde in 0.1M PB (350ml) until there was definite stiffening of the rat's body and

paraformaldehyde was running from the nose. To remove the brain, bone crushers were used to carefully remove any skull bone encasing the fixed brain and brainstem. The overlying dura layer was cut and removed using fine scissors. Before removal of the brain, a cut was made at the rostral end of the brain between the olfactory lobes and the cortex. The head of the rat was then turned upside down to allow the brain to fall naturally out of its position. Using fine scissors and working caudally to rostrally, any surrounding connective tissues were cut as the brain was increasingly freed from its position. At the rostral end, care was taken to delicately snip the optic tract as any damage to the optic tracts could result in loss of the SON. Finally, a small spatula was used to scoop the brain into a falcon tube containing post-fix soution (50:50 30% sucrose solution in 0.1M PB and 4% paraformaldehyde in 0.1M PB) in which the brain was left for 24 hours and then transferred into 30% sucrose solution for 24-48 hours or until the brain had sunk. Such post-fix solution acts in a dual manner to firstly cryoprotect the brain tissue. The sucrose solution acts to absorb any remaining water molecules in the tissue and so prevents any crystallization which is especially important when cutting fine sections of the brain on a freezing microtome. Secondly, the paraformaldehyde solution acts to form strong cross-linking aldehyde bonds throughout the tissue helping to preserve protein expression. The immersion of the brain in a second, more concentrated sucrose solution is an extra precautionary measure to avoid ice crystals forming and damage to the tissue. Using a sharp blade, a lateral cut was made at the caudal end of the brain to remove the unwanted brainstem tissue. Brains were immediately frozen in dry ice and stored at -70°C until use. Brain sections containing the MPN, SON and PVN were cut at 48µm on a freezing microtome and placed temporarily into petri dishes of 0.1M PB and then separated into three different vials containing cryoprotectant. Sections were cut at 48µm to allow more thorough and accurate quantitative analysis of the densely cell packed hypothalamic nuclei, particularly the SON. One vial was used for each immunocytochemical study to examine Fos or Fos and oxytocin expression. Once cut, brains sections were either stored in cryoprotectant at -20°C or used immediately.

2.6.2 Using Fos immunocytochemistry as a marker for neuronal activation

The expression of Fos (protein product of the immediate early gene *c-fos*) has been widely used to map neural circuitries subserving various forms of behaviour such as aggression and paternal

behaviour (Hasen and Gammie, 2005; Meddle et al, 2007; Haller at al, 2006). In addition, it has been extensively used to establish those neuroanatomical pathways that subserve male sexual behaviour in particular (Pfaus and Heeb, 1997). Specific copulatory parameters such as intromission and ejaculation are known to induce Fos expression in clusters of neurons in the SON, PVN, MPOA and subdivisions of the BNST and MeA of rats and birds (Balthazart and Ball, 2007; Bialy and Kaczmarek, 1996; Caquineau et al, 2006; Pattij et al, 2005; Parfitt and Newman, 1998; Pfaus and Heeb, 1997; Witt and Insel, 1994). Induction of Fos expression can be elicited via a range of numerous extracellular factors such as the activation of ion channels and the binding of neurotransmitters, excitatory amino acids and polypeptide fragments. Fos acts as a transcription factor by forming homomeric and/or heteromeric (with Jun) dimers which specifically bind to DNA sequence elements and influence transcriptional activity of certain proteins, namely AP-1 (activator protein 1) or CREB (cAMP responsive element binding) sites which subsequently activate their target genes. Thus, increased Fos expression may reflect permanent changes in neuronal re-modelling and neuronal plasticity (Bialy and Kaczmarek, 1996; Curran and Morgan, 1995).

Because Fos is expressed in the cell nucleus, Fos immunocytochemistry can be combined with neuropeptide immunocytochemistry to identify neuronal phenotype. In this instance, we used oxytocin to examine co-localisation of neuropeptide cytoplasmic labelling (oxytocin) with nuclear Fos protein expression. Such double immunocytochemical staining allows detection of discrete functional neurochemical pathways involved in the regulation of sexual behaviour.

To identify whether dopamine affected neuronal activation during male sexual behaviour, single Fos immunocytochemistry was performed and the number of Fos-positive cells were quantified to give a measure of activation in the relevant brain regions. Double immunocytochemistry for Fos and oxytocin was also performed and the number of Fos-positive oxytocin cells were counted, similarly. It is often debated that only one run of double immunocytochemistry for Fos and oxytocin is sufficient for quantification of Fos alone and co-localised Fos and oxyocin expression. However, it is thought

that during double immunocytochemistry and due to the additional vigorous washes, some of the Fos antiserum may be washed off and so some signal will be lost. When comparing the quantified Fos only expression after single and double immunocytochemistry in our studies, it was found that Fos only expression was consistently higher after single immnocytochemistry compared with double immunocytochemsitry (although only marginally and no differences between groups were observed). In addition, with single immunocytochemistry for Fos only, the brain sections tend to have lower background staining which allows for clearer and more accurate quantification. This was particularly important when analysing the densely populated SON.

2.6.3 Brain sections

As previously described, the MPN, SON and PVN are three brain nuclei that are believed to mediate consummatory aspects of male sexual behaviour. Thus, Fos and double Fos and oxytocin expression were immunocytochemically examined in these three brain areas.

Because the PVN is comprised of various parvocellular and magnocellular subdivisions, in addition to examining the PVN as a whole, each individual subregion within the PVN was also analysed separately (Figure 2.2).

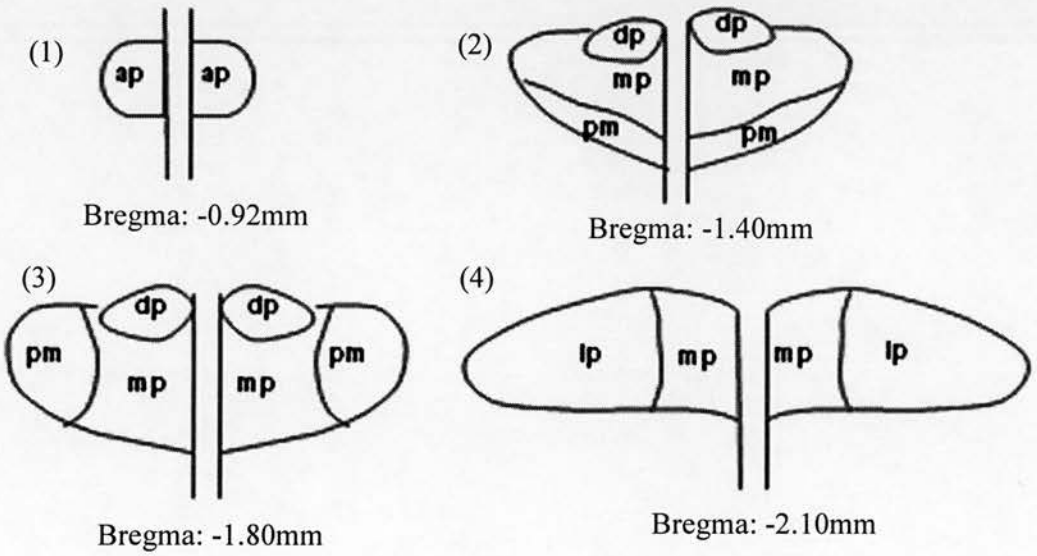


Figure 2.2: Subdivisions of the rat PVN examined, rostral-caudal (1-4). The coordinates are chosen according the rat brain atlas (Paxinos and Watson, 1988). ap = anterior parvocellular; dp = dorsal parvocellular; mp = medial parvocellular; pm = posterior magnocellular; lp = lateral parvocellular.

Thus, neuronal activation was analysed in each of the four hypothalamic levels and within each particular subregion as illustrated in Figure 2.2.

2.7 General principles of immunocyochemistry

Immunocytochemistry is the principal technique employed to detect the presence of antigens in tissue samples. Antigens are molecules that can elicit immune responses and these can include proteins, infectious agents, nucleic acids and polysaccharides. The fundamental premise of immunocytochemistry was first described by Coons et al in 1949 and over the years it has become a highly refined and versatile investigative tool used by many researchers. The concept of immunocytochemistry is the binding of an antibody to it's antigen in a tissue sample or medium followed by a labelling reaction to visualise the antibody-antigen interaction. Such labelling reactions can include an enzyme reaction or fluorescent dye to visualize the antibody-antigen complex. It is the surface of an antigen that interacts with the antibody, also known as the epitope. There are a variety of factors that can determine the success of antigen-antibody binding such as the location of the antigen in the sample, the location of an epitope, the antigen-detection method employed and the type and/or concentration of fixative used. To ensure good antibody binding and successful immunolabelling, it is vital that the antigenic epitope remains preserved and easily accessible to circulating antibodies.

Antibody molecules are glycoproteins consisting of four polypeptide chains (two identical heavy chains and two identical light chains) (see Figure 2.3). Antibody polypeptide chains are comprised of a variable domain (V) due to the variation in amino acid composition and one or three constant (C)

domains for the light and heavy polypeptide chains, respectively. The chains are held together by covalent and non-covalent bonds and form a stable, bilateral structure. It is the variable amino acid terminals at the end of both the light and heavy chain that constitute the antigen-binding sites (Figure 2.3).

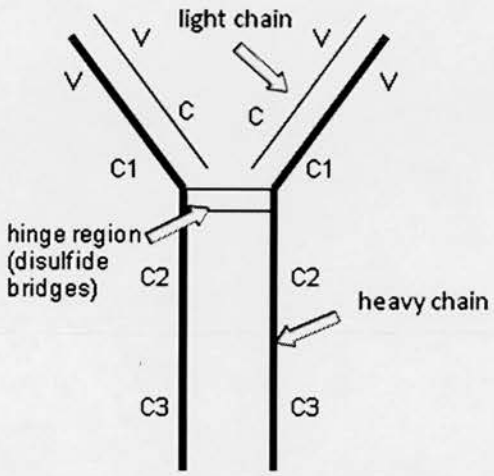


Figure 2.3: Diagram of an immunoglobulin. Immunoglobulins are comprised of light and heavy chains, each containing variable and constant domains. V=variable; C=constant; C1=constant region one; C2=constant region two; C3=constant region three.

Antibodies can be either monoclonal where they have only a single epitope site (antigenic binding site) or polyclonal where they have multiple epitopes and are directed towards various regions of the antigen molecule. An advantage of using monoclonal antibodies is that they have a single highly specific binding site for an antigen, thus they are less likely to bind to non-specific factors. However, if the epitope site becomes conformationally changed or damaged eg. during tissue fixation then the monoclonal antibody would loose all is antigenic properties and being reactive with only one antigenic site may involve fewer antibodies binding to the antigen, thus producing a weaker signal. Using polyclonal antibodies can overcome such a problem as they have multiple epitope sites. However, polyclonal antibodies can bind to non-specific sites in the tissue samples to produce "phantom" antigen-antibody complexes. A good antibody must have sufficient avidity and affinity properties to ensure that the interaction between the antigen and antibody is relatively stable and that

the paratropic binding of the antibody to the antigenic epitope is strong enough (good stereochemical compatibility). High avidity is a necessary component of a good antibody as it helps to prevent any antibody being washed off the tissue samples during the series of immunocytochemical washes and staining processes.

Visualisation

Visualisation of the antigen-antibody complex can be performed with visible labelling such as an enzyme marker like horseradish peroxidase which reacts with a substrate and a chromagen to produce a coloured precipitate that can be viewed with a light microscope. For this reaction to occur endogenous peroxidase present in cells such as macrophages must be blocked to reduce non-specific background staining. Thus, prior to the reaction, tissue sections are washed in a hydrogen peroxide solution containing methanol which inhibits formation of endogenous peroxidase. The coloured end-product is visualised using the common diaminobenzidine (DAB) (supplier details noted on P.91) method, which acts as a peroxidase substrate. The horseradish peroxidase binds to a pre-formed complex of avidin and biotin aptly named the avidin-biotin peroxidase complex (ABC) (Figure 2.4). Such a complex binds to the previously bound biotinylated secondary antibody and acts to amplify the antigen-antibody signal.

When performing double immunocytochemistry, the DAB reaction can involve the addition of nickel ammonium sulphate (to produce a black precipitate) or not (to produce a brown precipitate). The chromagen, DAB is usually a colourless substance which acts as a substrate for the peroxidase reaction. The DAB solution contains glucose and glucose oxidase, thus the actions of glucose oxidase result in the release of an oxygen atom which then reacts with water to form hydrogen peroxide. This newly formed hydrogen peroxide reacts with DAB to form an instable DAB/peroxidase complex. The horseradish peroxidase catalyses the oxidation of DAB by hydrogen peroxide to produce an insoluble black granular precipitate, which is enhanced in the presence of nickel ammonium sulphate.

Figure 2.4: Diagram of the avidin-biotin complex (ABC) method

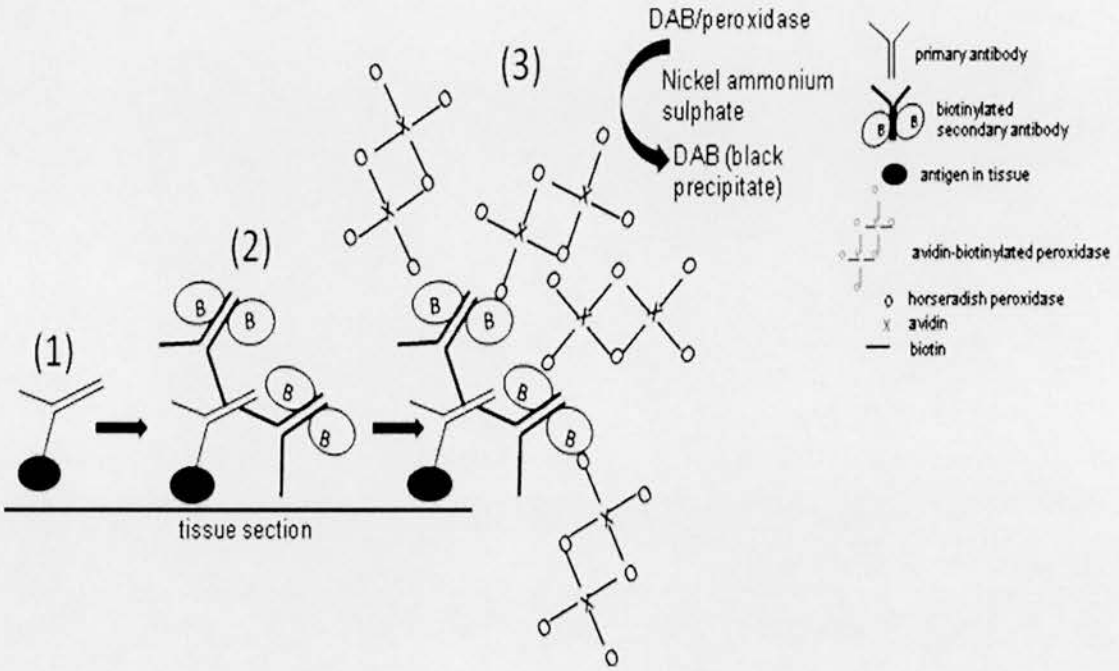


Diagram of the avidin-biotin complex (ABC) method. (1) The primary antibody (rabbit anti-rat when using rat sections) binds to the antigen present in the tissue. (2) The biotinylated secondary antibody (goat anti-rabbit) subsequently binds to the primary antibody. (3) The pre-formed avidin-biotinylated peroxidase complex (ABC) then binds to sites on the biotinylated secondary antibody. The chromagen DAB is oxidised by the horseradish peroxidase complex to produce a black precipitate, a reaction which is enhanced by the presence of nickel ammonium sulphate.

Adapted from Pollak and Van Noorden, 2003

2.7.1 Protocol for Fos immunocytochemistry

Ninety min after drug injection (penile erection study) or being placed with a receptive female (intromission study), rats were deeply anaesthetised and transcardially perfused as described previously. The transient increase in Fos protein expression in response to neuronal stimulation, is generally observed between 30 and 60 min, with levels returning to half the maximal value at 120 min (Kaczmarek, 2002; Vosatka, 1989). Most sexual behaviour studies using Fos immunocytochemistry to functionally map CNS involvement, generally use a 60-90 time period for Fos protein induction after copulation (Pfaus and Heebs, 1997; Veening and Coolen, 1998). Thus, 90 min appears to be an appropriate time period to allow for maximal Fos protein expression when investigating dopamine ligand-mediated control of penile erection.

Solutions for perfusion fixation:

1. Heparinised Saline(0.9%): 1L dH_20 + 0.9% NaCl (cat nos. S7653, Sigma, UK) + 0.0129% heparin (cat nos. H0777, Sigma, UK).
2. 4% Paraformaldehyde in Phosphate Buffer (PB) (pH 7.3-7.4): For 1L, 500ml dH_20 + 4% paraformaldehyde (cat nos. P6148, Sigma, UK). NaOH pellets (cat nos. 480878, Sigma, UK) were added to the solution to aid salt dissolution) + 500ml 0.2M PB.
3. Post-fix solution (4% paraformaldehyde + 15% sucrose in 0.1M PB): For 100ml, 15% sucrose (cat nos. S0389, Sigma, UK) / 100ml 4% paraformaldehyde in 0.1M PB.
4. 30% sucrose solution: For 1L, 30% sucrose / 1000ml 0.1M PB
5. Cryoprotectant to store brain sections at -20°C: For 1L, 20% glycerol (cat nos. 10484K, VWR, UK) + 30% ethylene glycol (cat nos. 24407.326, VWR, UK) + 500ml 0.2M PBS (phosphate buffered saline, for 1L, 0.2M PB + 0.9% NaCl).

Solutions for immunocytochemistry:

1. 1M phosphate buffer (PB) (pH 7.35-7.4): for 1L, add 11.5% disodium hydrogen orthophosphate (cat nos. S/4440/53, Fisher Scientific, UK) + 2.72% sodium dihydrogen orthophosphate (cat nos. S/3770/53, Fisher Scientific, UK) to 1000ml dH_2O.

2. 0.1M PBT (0.1M PB + 0.2% Triton X-100): for 1L, add 2ml Triton X-100 (cat nos. 437002A, VWR, UK) to 1000ml 0.1M PB.
3. 0.1M phosphate buffered saline (PBS): for 1L, add 0.9% NaCl to 1000ml 0.1M PB.
4. 0.2M sodium acetate buffer: for 1L, add 1.64% sodium acetate (anhydrous) (cat nos. 102365R, VWR, UK) to 1000ml dH2O, pH to 6.0 with acetic acid.
5. Glucose oxidase-Nickel-DAB solution: for 100ml (comprised of two solutions), solution one; 50ml 0.2M PB + 2.5g Nickel II sulphate (cat nos. 83751.230, VWR, UK) + 0.4g glucose (cat nos. G7528, Sigma, UK) + 0.08g ammonium chloride (cat nos. 100173D, VWR, UK), solution two; DAB (cat nos. D8001, Sigma, UK) (25mg/ml) aliquot is added to 49ml dH_2O and filtered before adding to solution one. Immediately prior to use 0.003g glucose oxidase (cat nos. G6641, Sigma, UK) is added to the Nickel-DAB solution.
6. Hydrogen peroxide DAB solution: for 100ml (comprised of two solutions), solution one; 50ml 0.2M PB + 100µl hydrogen peroxide (cat nos. 316989, Sigma, UK), solution two; DAB (25mg/ml) aliquot is added to 49ml dH_2O and filtered before adding to solution one.
7. Gelatine (for subbing slides): for 1L, add 5g gelatine (cat nos. 48723, Sigma, UK) + 0.5g chromium (III) potassium sulphate-12 hydrate (cat nos. 243361, Sigma, UK) to 1000ml warmed dH_2O, allow to cool and store at 4°C.

2.7.1 Fos immunocytochemistry

Each vial containing brain sections with the MPN, SON and PVN were drained and separated from cryoprotectant. Sections were briefly immersed in 0.1M PB prior to draining using small aluminium baskets, placed on a shaker (70 r.p.m.) at room temperature and initially washed in 0.1M PBT (2x5 and 2x 10 min) and then 0.1M PB (1x 5 min). This was replaced with 20% methanol and 1% H_2O_2 in 0.1M PB for 15 min to quench endogenous peroxidase activity in the tissue. The sections were washed with 0.2M PBT (2x 7.5 min) and 0.1M PB (1x 5 min) which was then replaced with 1% normal sheep serum (NSS) (cat nos. 3772, Sigma, UK) in PB-T for 1 hr at room temperature (r.t.), which acts as a pre-incubation block and helps to reduce the non-specific staining in sections. Sections were then incubated in vials containing rabbit anti-rat polyclonal anti-Fos antibody (1:1000, cat nos. 624401, Cambridge Bioscience, UK), 1% NSS, 0.1M PB-T for 24h at 4°C. The following day the sections were washed in 0.1M PBT (3x5 and 2x10 min or 8x4 min for double labeling). The

sections were incubated in a secondary antibody solution containing biotinylated goat anti-rabbit IgG, normal goat serum (cat nos. PK-6106, Vectastain Elite, Vector Laboratories, Inc.) and 0.1M PBT for 1h at room temperature. The sections were washed again in 0.1M PBT (3x5 mins) prior to incubation with Avidin DH and biotinylated horseradish peroxidase (ABC Kit, Vector) and 0.1M PBT for 1 hr at RT°C. Tissue sections were then further washed in 0.1M PBT (2x10 mins) and 0.1M sodium acetate buffer (pH6) (1x5 min).

Fos labeling was visualized using the DAB (diaminobenzamide) method. For this method two solutions were initially made; Nickel II sulphate, glucose and ammonium chloride were added to 0.2M sodium acetate buffer and DAB (25mg/ml) was added to dH_20. The DAB solution was filtered before being added to the Nickel II sulphate solution and glucose oxidase type Vii-S was added immediately prior to use. The sections were immersed in the above visualisation solution and closely monitored to observe the reaction which took approximately 5-7 mins to occur. During this time, a control section was briefly removed from the DAB solution and viewed under a light microscope to confirm progress of the DAB reaction. Fos labelling was seen as a solid black nuclear precipitate. Sections were then washed with 0.1M sodium acetate buffer for 5 minutes and then rinsed in 2x5 and 1x10 min wash in 0.1M PB.

Brain sections were mounted on gelatinised slides and dehydrated through increasing concentrations of ethanol (50%, 70%, 90%, 95% for 5 minutes) and through 100% ethanol 2x10 min before being washed in xylene twice for another 10 min. The sections were finally coverslipped (Depex, BDH) and allowed to dry.

2.7.2 Fos and oxytocin immunocytochemistry

Double immunocytochemistry for Fos and oxytocin was performed on free-floating coronal sections cut from the same brain regions and at the same thickness as detailed above. The sections were processed in the same way as described above, however, after Fos visualisation, the sections were placed in 2x5 mins PB-T washes and this was replaced with a 0.3% H_2O_2 wash for 15 mins. The sections were placed in a further 2x10 min PB-T washes and then incubated in a solution containing

polyclonal antiserum against oxytocin (cat nos. PC226L, 1:1000; Calbiochem, UK), 1% NSS and PB-T for 24h at 4°C. The sections were washed in PB-T for 3x10 min and then incubated in the secondary biotinylated antibody and then in the avidin-biotin complex using ABC kit as described above. Sections were washed in 0.1M PB for 5 min and visualisation was achieved using a solution composed of DAB, 0.2M PB and H_2O_2 resulting in brown cytoplasmic labelling within 1 min. Sections were washed in 6x5 min 0.1M PB washes before mounting on gelatinised slides and dried through alcohols as described above.

Controls

Although studies using the above Fos and oxytocin antiserum have been published previously in our lab (Brunton et al, 2006; Caquineau et al, 2006), steps were taken to confirm the effectiveness and specificity of the Fos and oxytocin antiserum. The following negative controls were carried out:

Fos

(1) Omission of the primary antibody – 0.1M PBT and 1% NSS

Oxytocin

(1) Omission of the primary antibody – 0.1M PBT and 1% NSS
(2) Substitution of primary antibody with NRS – 0.1M PBT, 1% NSS and NRS (1:1000 dilution).

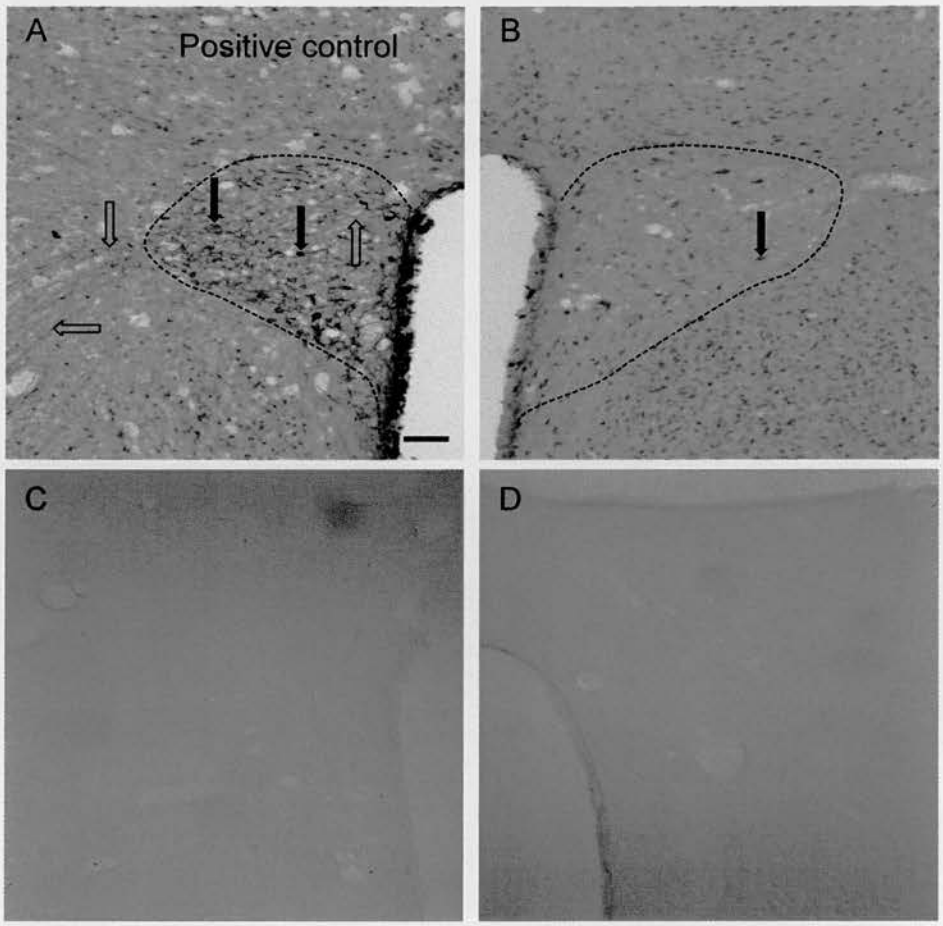
It can be seen that with omission of either primary antibody, no labelling was detected in any of the tissue sections. With substitution of the oxytocin antibody with NRS, there was very little non-specific staining in the PVN (Figure 2.5), suggesting that there is minimal background staining associated with application of the oxytocin antisereum

2.7.3 Immuno labelled cell quantification

Fos

Using a light microscope (x25 magnification) and coded slides, Fos-positive cells were counted as those with black nuclear staining in the MPN, SON and PVN. Because the MPN was not as clearly defined as the SON and PVN, nearby neuroanatomical landmarks (the anterior commisure and fornix,

Figure 2.5: Negative controls for Fos and oxytocin antiserum



Labelling of oxytocin antiserum in the PVN (A). Negative controls for oxytocin antiserum are shown in B (substitution of oxytocin antibody with NRS) and C (omission of oxytocin antibody). Negative control for Fos antiserum is shown in D (omission of Fos antibody). Filled arrows indicate oxytocin-positive cell bodies and empty arrows show oxytocin-positive fibres. Scale bars represent 100μm.

see Figure 1.3) were used as reference regions when analysing each MPN region. This helped to maintain consistency when measuring Fos expression in the MPN. For the MPN and SON, approximately 2 and 4 sections per rat, respectively, were counted. For the PVN, Fos-positive cells were quantified in 4 sections per rat in the following PVN subdivisions; anterior parvocellular, dorsal parvocellular, medial parvocellular, posterior magnocellular and lateral parvocellular (ap, dp, mp, pm and lp) and then summed together to give a value for the PVN as a whole. All values were averaged per rat and then per group.

Fos and oxytocin

For quantification of Fos and oxytocin cells, the counts were performed as described above. A Fos-positive oxytocin cell was counted as a cell with black nuclear staining surrounded by brown cytoplasmic labelling. In addition, to Fos only counts however, the total number of oxytocin cells and the number of co-localised oxyocin cells with Fos protein were quantified separately in the each of the three brain nuclei. The number of Fos-positive oxyocin cells were expressed as a percentage of the total number of oxytocin cells.

2.8 Immunofluorescence – colocalisation of D2-like dopamine receptors with oxytocin neurons

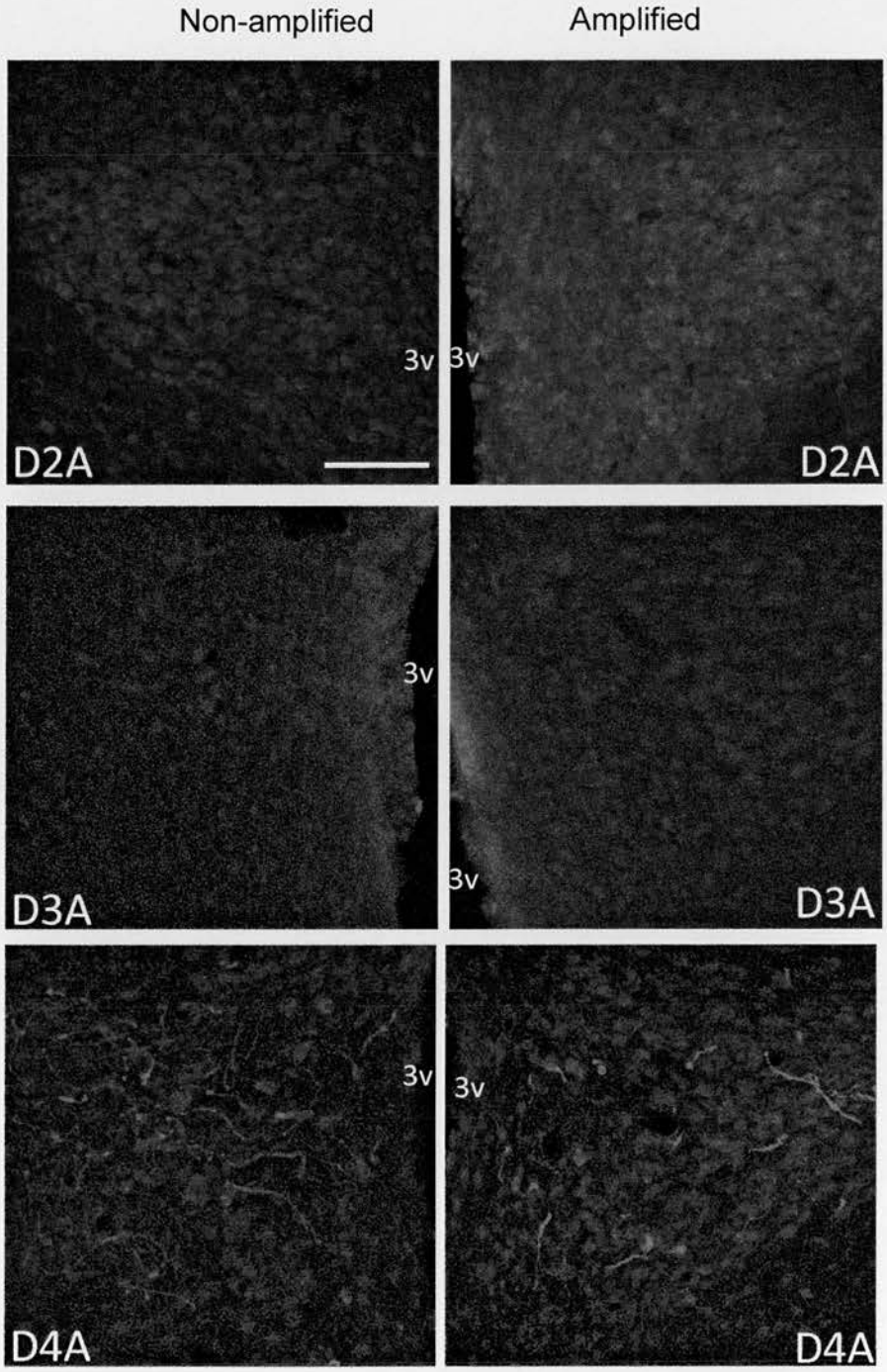
The double immunofluorescence technique was chosen to investigate co-localisation of D2-like receptors and oxytocin because (1) receptor labelling can give a much weaker signal in comparison to neuropeptide labelling, thus using fluorescently labelled molecules to bind to the dopamine receptor primary antibody would show fluorescently labelled receptor protein against a black background and so make the labelling comparatively easier to visualise than using the Nickel-DAB method and (2) double immunofluorescence enables the visualisation of more than one fluorescently labelled molecule in the same cellular compartment. Thus by altering the excitation light (light of longer wavelength), one can change the fluorescent signal emitted and so distinguish two or more fluorescently labelled areas of the sample. This is especially important when looking for co-localisation as, a confocal microscope will capture each fluorescently labelled image separately and

then overlay the two fluorescent images thus making it easier and clearer to visualise co-localisation sites.

Dopamine receptor immunofluorescence was initially perfomed without an additional amplification stage, termed direct immunofluorescence. Here, brain sections were washed in 0.1M PB and 0.1M PBT prior to incubation in the blocking serum. However, the receptor signal and cellular resolution was difficult to visualise due to high levels of non-specific background labelling. Thus, it was decided to introduce additional endogenous biotin blocking steps, termed indirect immunofluorescence. Here, prior to incubation in the blocking serum, the brain sections were firstly immersed in a medium containing unlabelled streptavidin which binds to endogenous biotin in the tissue sample. The sections were then exposed to unlabelled biotin which in turn blocked streptavidin sites. The normal blocking and labelling-steps were then performed as with the direct method. As can be seen in Figure 2.6, when comparing non-amplified and amplified dopamine receptor immunolabelling; there is greater dopamine receptor signal intensity for the D2, D4 and to a lesser extent for the D3 receptor (cat nos. 324393, 324402, 324405, all rabbit polyclonal IgG, used at 1:1000, 1:3000 and 1:1000 dilution respectively, all Calbiochem, UK).

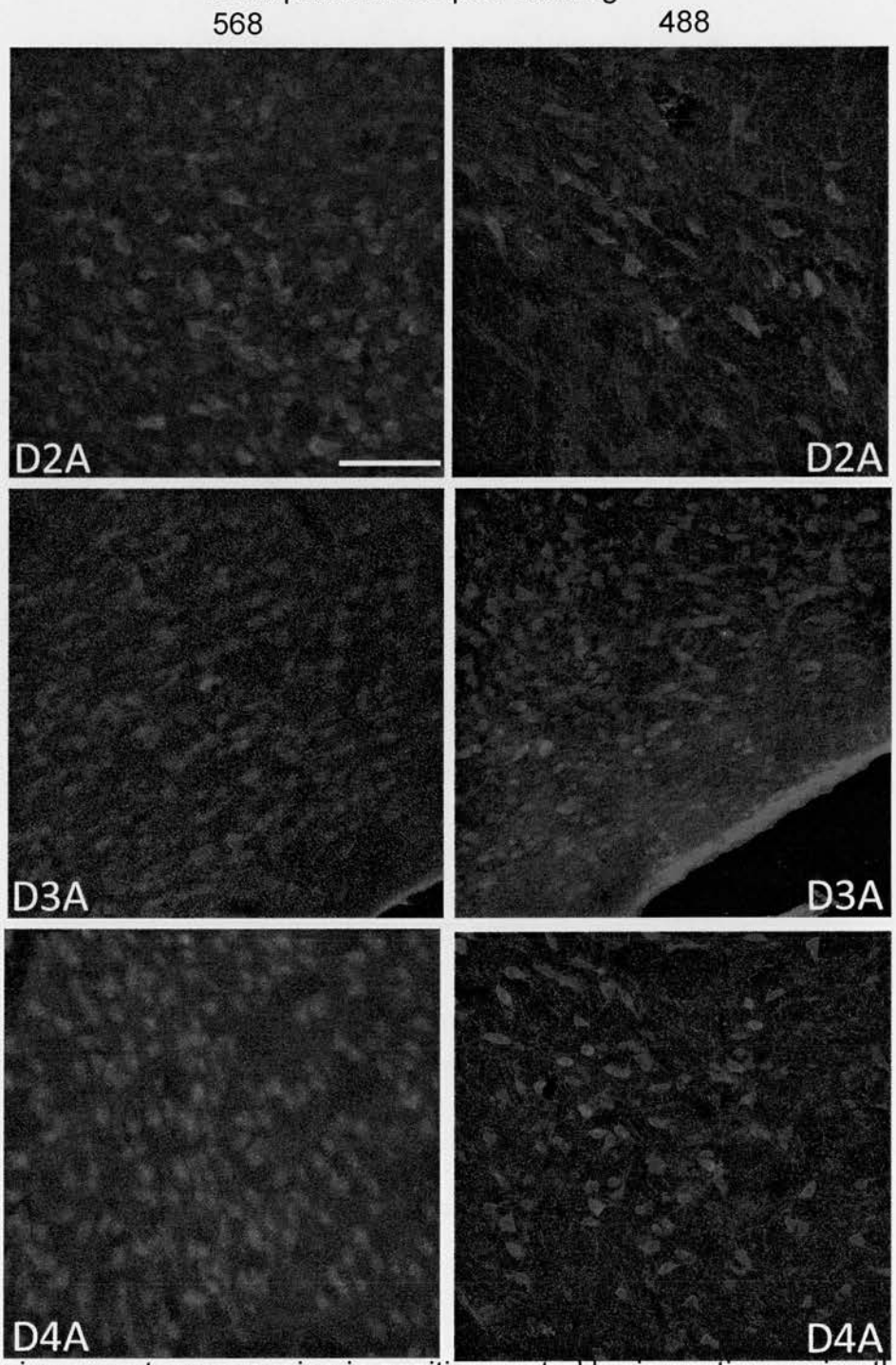
The Alexa-fluor fluorescent secondary antibody 568 (red) was initially used to visualise dopamine receptor expression, however, it was decided that the Alexa-fluor 488 (green) was the better fluorophore to use for the dopamine receptor signal (Figure 2.7) due to the natural, inherent brightness of the 488 fluorophore compared to the others.

Figure 2.6 : Non-amplified and amplified dopamine receptor staining



Non-amplified and amplified dopamine receptor staining for the D2, D3 and D4 receptor in the PVN. (Scale bar is the same for all images and represents 100μm). It is clear that there is greater dopamine receptor signal intensity (particularly for the D2 and D4 receptor) with the amplification stage.

Figure 2.7 : The use of Alexa-fluor 488 versus Alexa-fluor 568 for dopamine receptor staining



Dopamine receptor expression in positive control brain sections using the Alexa-fluors 568 and 488 in the striatum for the D2 and D4 receptors and in the olfactory tubercle for the D3 receptor. (Scale bar is the same for all images and represents 100μm). The Alexa-fluor 488 stained sections show brighter staining with clearer cellular resolution.

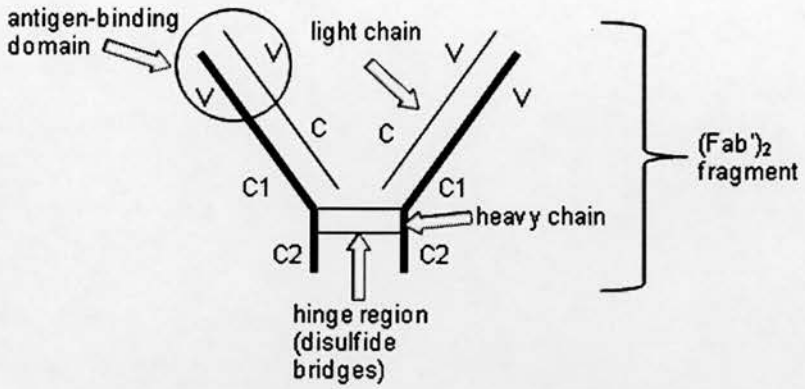


Figure 2.8: Structure of $(Fab')_2$ fragment of an Alexa-fluor antibody. $F(ab')_2$ contains the antigen-binding domains and only a small proportion of the constant heavy chains.

For the fluorescently labelled secondary antibodies, $F(ab')_2$ fragments of the antibody were used as opposed to the entire immunoglobulin. The $F(ab')_2$ fragments are comprised of antigen-binding sites, bound at the hinge through disulfide bridges (Figure 2.8). They are void of most, but not all, of the immunoglobulin constant regions. $F(ab')_2$ fragments have high avidity and are the preferable choice for secondary detection because interactions with the constant-regions of the antibody with receptor-bearing membranes is prevented due to the absence of these antibody domains. Thus, using these fragmented immunoglobulins helps to reduce the extent of non-specific background labelling and so improve cellular resolution when visualising.

2.8.1 Protocol for dopamine D2-like receptor and oxytocin immunofluorescence

Brains were cut on the microtome as described above. Washes were all done on shakers at room temperature unless stated otherwise. Using a 6x4 multi-well plate, 1-2 sections were placed in several chambers and washed in 4x15 min 0.1M PB washes. Using the biotin blocking kit (Oncogene Sciences, UK), brain sections were immersed in 3-4 drops of reagent A for 30 min and this repeated for reagent B followed by 4x10 min washes in 0.1M PB. Sections were then incubated in 1% BSA

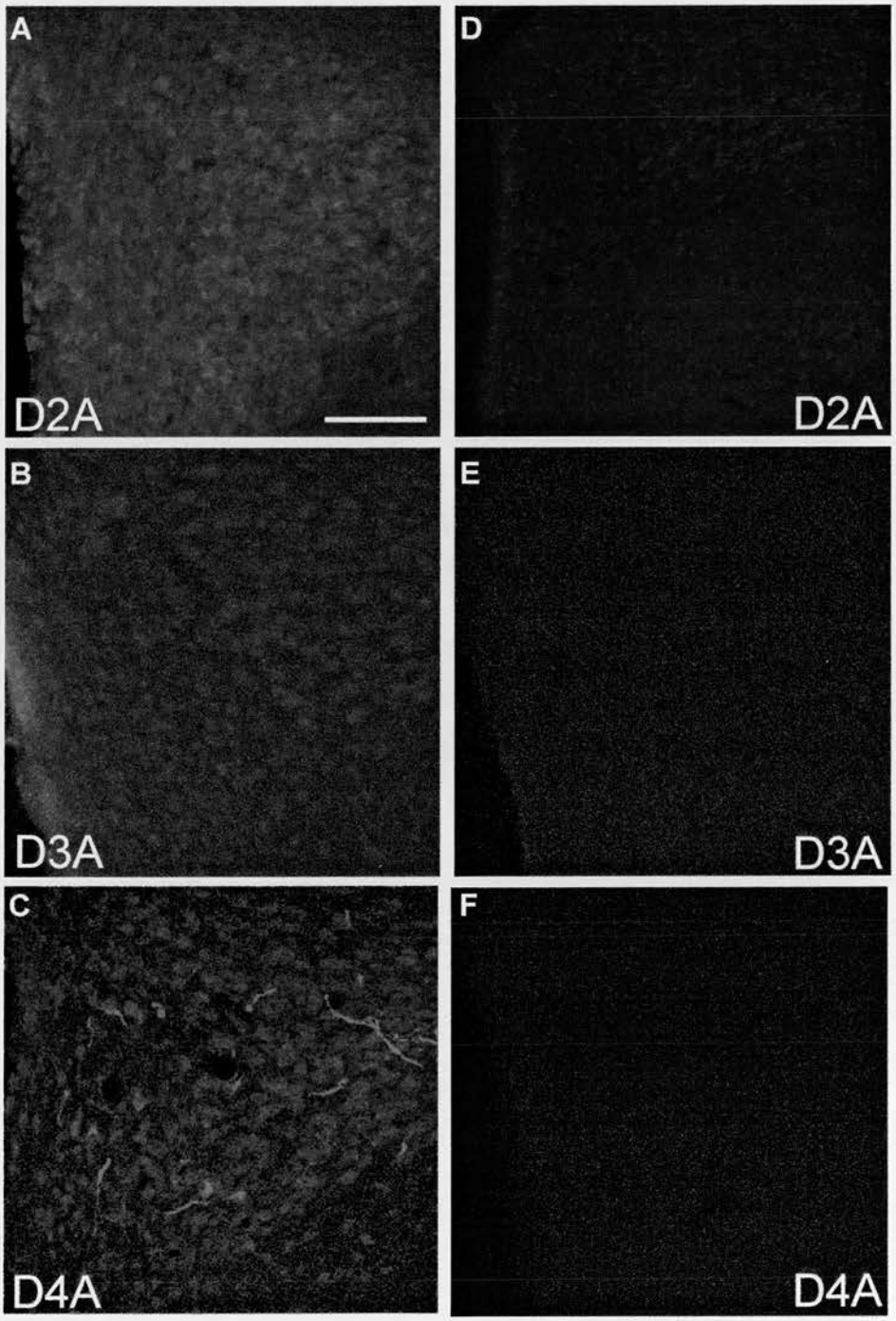
(bovine serum albumin, Sigma, UK) in 0.1M PB-T overnight at 4°C. The following day, sections were co-incubated with anti-D_2, anti-D_3 or anti-D_4 and anti-oxytocin (cat nos. MAB5296, mouse monoclonal IgG 1:1000, Chemicon, UK) antibodies in 1% BSA in 0.1M PB-T for 48 h at 4°C. After 4x10 min washes in 0.1M PB-T, sections were washed with a secondary biotinylated anti-rabbit IgG (1:1000, Vectastain Elite, Vector Laboratories, UK) in 1% BSA and 0.1M PB-T for 1h. After another 4x10 min 0.1M PB-T washes, sections were co-incubated with Strepavidin-Alexa Fluor 488 goat anti-rabbit (for all dopamine receptors, cat nos. A11070, 1:1000, Invitrogen, UK) and Strepavidin-Alexa Fluor 568 goat anti-mouse (for oxytocin, cat nos. A11019, 1:1000, Invitrogen, UK) in 1% BSA and 0.1M PB-T for 1 h. From this point, all sections were protected from light. Sections were washed in 0.1M PB for 3x10 min before mounting onto slides using 0.05M PB and Molviol. Once dried slides were wrapped in foil and stored at 4°C to preserve the immunofluorescence signal. Photomicrographs were taken using a Zeiss confocal microscope.

Autofluorescence (fluorescence emitted from non-specific cell proteins present in sample) can often make it difficult to distinguish between specific fluorescence signal and background staining. This emitted signal is due to endogenous flourophores within certain cells and most of the autofluorescence originates from intracellular structures such as mitochondria and lysosomes. The following negative controls were included to see the specificity of the primary antibody and to establish the extent of autofluorescence in the sample:

(1) removal of primary antibody from incubation medium.
(2) substitution of primary antibody with immune serum from the species the primary antibody was raised in.

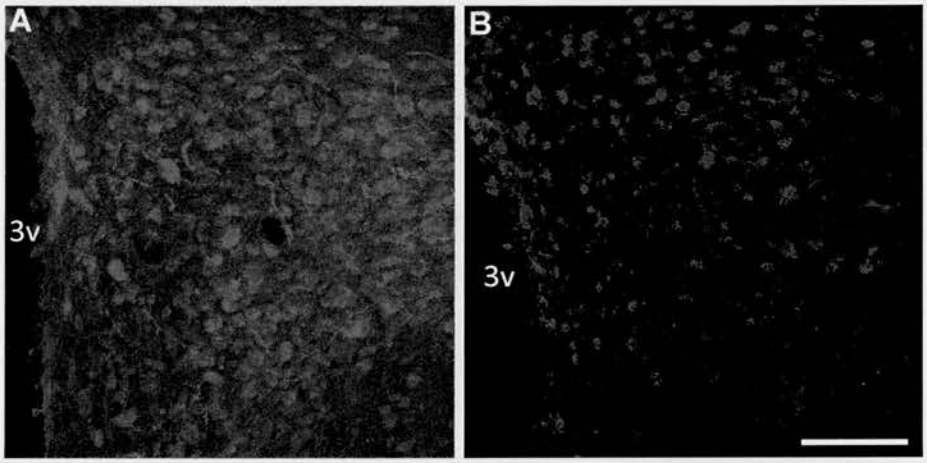
Omission of the dopamine receptor antibody resulted in a complete loss of immunofluorescence signal (Figure 2.9). The same effect was also observed with the oxytocin antibody. Additionally, substitution of the primary antibody with pre-immune serum produced relatively low levels of non-specific staining (Figure 2.10).

Figure 2.9 : Negative controls for dopamine receptor antiserum (1)



Dopamine receptor expression in the PVN (A,B,C) compared to the negative control (omission of primary antibody) (D,E,F). (Scale bar is the same for all images and represents 100μm). Little or no receptor staining can be seen in the the negative control sections.

Figure 2.10 : Negative controls for dopamine receptor antiserum (2)



Dopamine receptor expression in the PVN (A) compared to the negative control (omission of primary antibody and substitution with normal rabbit serum) (B). (Scale bar is the same for all images and represents 100μm).

Photobleaching can occur when the fluorescently labelled specimens are exposed to high intensities of excitation light. The fluorescent signal can fade and eventually become non-flourescent (this is thought to be due to in part oxidisation of fluorescent molecules Becker et al, 1996; Chen et al, 1995). To reduce the effects of photobleaching sample sections were permanently wrapped in foil and exposure to light was minimised.

2.8.2 General principles of confocal microscopy

Confocal microscopy was first established by Marvin Minky (1 Minky Memoir on inventing the confocal microscope, Scanning) in the 1950s and principally it allows the creation of highly resolved images that would otherwise appear blurred using conventional microscopes. Confocal microscopes act to transmit in-focus light and reject out-of-focus light, thus creating a much sharper and more contrasting image. During scanning of a specimen, only one point of the sample is illuminated at a time, allowing constructing of 2D and 3D images pixel by pixel. Thus using the vertical axis, a 3D image of the specimen can be constructed by combining a series of thinner slices.

The choice of objective lens and numerical aperture (light-gathering ability) are extremely important elements in confocal microscopy. Signal intensity is dependent on the numerical aperture used (signal intensity increases with numerical aperture) and numerical aperture is dependent on the objective used (higher objectives have greater numerical apertures). Additionally, the pinhole diameter is equally important, as too small a pinhole size may reduce the number of fluorescent emissions reaching the detector, likewise if the pinhole diameter is too large then the light intensity may be too great and lead to photobleaching and degradation of the fluorophore. As previously stated, the pinhole aperture acts to prevent out-of-focus light entering the focal plane, however, it strongly determines the thickness of the optical sectioning (emitted light form sample slice collected by detector)

Fluorophores are molecules that are activated by high energy (excitation) light of a particular wavelength. Fluorophores absorb the light and become highly excitable, however, they loose energy very quickly due to molecular collisions and so exhibit a relatively lower energy state. The lower excitable state involves the fluorophore emitting light of longer wavelength than the excitation light (Figure 2.11). Thus, when a fluorescent sample is illuminated with blue excitation light for example, the fluoresced light is green.

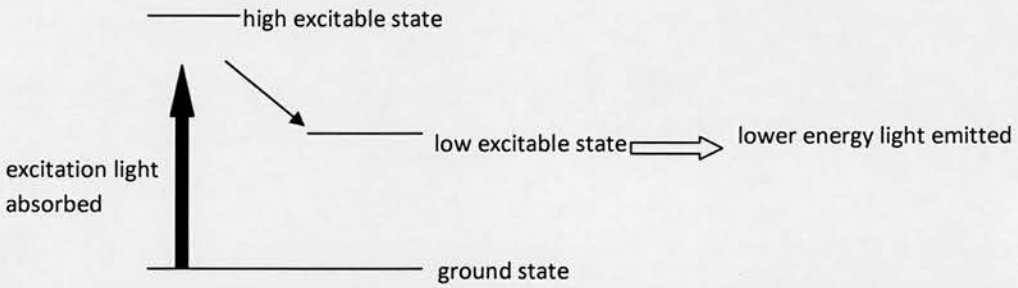


Figure 2.11: Emission of fluorescence light

When light (excitation) of a certain wavelength is shined on a sample, the excitation light is reflected off a dichroic mirror and onto the fluorescent specimen. Emitted light from the sample is then transmitted through the dichroic mirror via a pinhole and is measured by a detector. Only light of longer wavelengths are transmitted whilst light of shorter wavelengths are reflected, thus the experimentor should see emitted light from the sample and not other generated scattered or non-specific light (Figure 2.12).

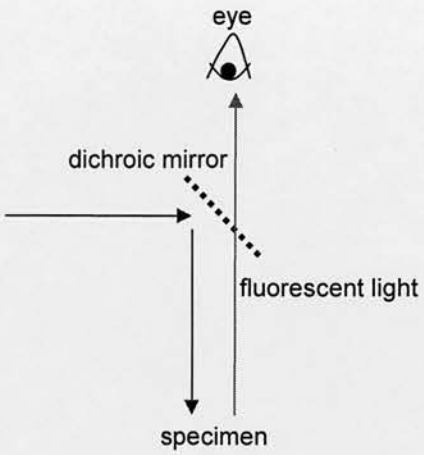


Figure 2.12: Simplified version of how a fluorescence microscope works. Light of a certain wavelength (in this case blue) is reflected off a dichroic mirror and onto the specimen. Fluorophores in the specimen adsorb this light and transmit emitted light of lower energy (in this case green).

When using confocal microscopy, steps must be taken to reduce technical errors and the creation of false positive results. Light reflected and light transmitted by dichroic mirror and pinhole is not always 100% efficient. Thus emissions of one fluorophore may be detected in the channel of another fluorophore and so obscure the visualisation of captured images, this is termed bleed-through. To reduce the likelihood of bleed-through the following measures were performed;

(1) single labelled controls were used and using single-tracking on the confocal laser, (where only one laser is activated at a time), this allowed the detection of any signal (cross-talk or bleed-through) received in the alternate channel.

(2) sequential scanning (multitracks) on the confocal laser was used - here the laser in one channel is activated and emission light is collected before the laser in the second channel is activated and emission light is collected, thus reducing any excitation / emissions overlap between the respective channels.

Once under the confocal microscope, an optimal and accurate pinhole size and thickness of optical slice was chosen so as to minimise exposure of the sample to high intensity light. The pinhole size determines the thickness of the specimen slice from which emitted light is collected (optical section). A small pinhole diameter produces thinner optical sections and gives better Z axis resolution; however, the signal intensity can be decreased. The same optical thickness was used for all channels. Included in the confocal software is a facility called "range indicator" which establishes more objective acquisition settings and so reduces the subjective creation of high contrast, saturated images by the experimenter.

2.8.3 Analysis of co-localisation

The main purpose of performing the dopamine receptor and oxytocin immunofluorescence study was qualitative and not quantitative. We primarily wanted to establish an anatomical link between central dopamine receptors and oxytocin by looking for dopamine D2-like receptor and oxytocin co-localisation in the MPN, SON and PVN. Double-labelled immunofluorescence images were captured via sequential scanning (as described before) on a Zeiss confocal microscope.

2.9 Behavioural techniques

2.9.1Female receptivity

As previously described, OVX sexually receptive females were used in the intromission study. After s.c. injection of progesterone and oestrogen 48h and 4h, respectively prior to experiment day, the female rats were placed with virgin males to check for sexual receptivity. Only those females displaying proceptive behaviour such as jumping, darting and ear wiggling and receptive behaviour such as lordosis (where the female extends her body and arches her back to allowing the mounting male to intromit) on experiment day were used in the study.

2.9.2Male sexual experience

In the intromission study, sexually-experienced male rats were used to ensure they were capable of copulation. Sexual experience was gained by placing a male with a receptive female over 2-3 nights; copulation through to ejaculation was confirmed by the presence of a vaginal plug of semen. This was repeated twice to allow 2 runs of sexual experience. Those males who did not mate after 4 days of being placed with a female were excluded from the study.

2.9.3 Penile erection observations

Rats were placed individually into Plexiglas cages (30cm x 30cm x 30cm) during observation and recording of behaviour under normal light. After drug injection the number of stretching, yawning and penile erection episodes were counted for 60 min in sexually naive rats. Penile erection was scored when the penis emerged from the penile sheath, accompanied by penile grooming and hip flexion.

2.9.4 Intromission observations after placement with a receptive female

Male rats were placed into the transparent Plexiglas cage (30cm x 30cm x 30cm) of a receptive female during observation and recording of behaviour. All behavioural experiments were conducted in dark red light. Males were randomly assigned to two separate groups: those placed alone in a novel environment (clean cage with bedding) with no female or female cues (ALONE) and those placed with a receptive female. During the test period, it became apparent that some rats would display mounting but not intromitting behaviour. Thus, the paired males comprised two groups; males that did not intromit (NI) and those that did intromit (I). A drug was given 15 min prior to the male being placed with the receptive female. Once placed in the cage with the female and via remote viewing, the following copulatory parameters were recorded in each male rat for 15 min; mount and intromission latency (ML and IL) and mount and intromission frequency (MF and IF). Mount and intromission latency define the times taken for the male to display his first mount and intromission from when he is initially placed with the receptive female. Mount and intromission frequencies define the number of mounts and intromissions achieved prior to ejaculation during the 15 min being placed with the receptive female. Mounts were scored when the male was situated directly behind the female

and placed his front paws on the female's caudal back but did not show pelvic thrusting when the female rat displayed lordosis. Intromissions were scored when the male successfully mounted the female directly from behind and displayed a series of pelvic thrusts whilst the female expressed lordosis. Behavioural analysis was conducted by individuals who were on most occasions blind to the treatments administered.

2.10 Intracavernosal pressure (ICP) analysis

2.10.1 General principles

Measurement of intracavernous pressure (ICP) as an index of erectile function has been frequently used in previous studies and allows for a direct measurement of penile erection in anaesthetised rats (Allard et al, 2002; Chen et al, 1999; Giuliano et al, 2001) and various other species (Hayes and Adaikan, 2002; Lavoisier et al, 1990; Lin et al, 1996; Mizusawa et al, 2002 for review). During erection, the penis becomes engorged with blood and by placing a cannula attached to a pressure detector in one corpus cavernosum, measurement of intracavernosal pressure serves as a consistent and accurate physiological marker of penile erection. The ICP model has been used to investigate control of penile erection in various sexual contexts such as, pharmacologically-induced erection in isolated males, non-contact erections, reflexive erections and copulation-based penile erection (Bernabe et al, 1995, 1999; Chen et al, 1999; Giuliano et al, 1994). The sexual context that elicits the most powerful increases in ICP is that associated with copulatory behaviour (Giuliano et al, 1994; Bernabe et al, 1999) where mounting, intromitting and ejaculation progressively increase ICP responses. This is to be expected as in a mating environment with a receptive female, the male rat will receive olfactory and sensory cues which will stimulate multiple pathways that converge on those autonomic circuitries controlling erection. However, it is important to add that transient and robust increases in ICP can also be seen in anaesthetised rats under a range of experimental conditions including after injection of known pro-erectile agents (Allard at al, 2002; Chen et al, 1999; Hayes and Adaikan, 2002; Kimura et al 2006), upon electrostimulation of peripheral penile nerves (Chen et al, 1999; Martinez-Pineiro et al, 1994; Sezen and Burnett, 2000; Xing et al, 2005) and after electrical activation of known brain nuclei influencing penile erection (Chen et al, 1992; Chen et al 1997; Giuliano et al, 1996; Sato and Christ, 2000). The anaesthetised preparation is a useful *in vivo* model

because in most cases, physiological responses (albeit to a lesser degree in some cases) are maintained. Thus, the ICP model proves to be an extremely refined investigative tool when trying to establish particular neural pathways underlying context-specific penile erection. In our study we are using the ICP model to investigate the pathways recruited by the non-selective dopaminergic agonist, apomorphine. The surgical procedures involved in this experimental set up have been previously described in section 4.

2.10.2 Analysis of ICP recordings

ICP tracings were analysed retrospectively using computer software in the Pfizer laboratory. ICP increases were detected as rises above a certain threshold value; this value was calculated as the ICP value averaged over the 10 min baseline recording before drug injection plus two standard deviations. These rises in ICP served as physiological markers of penile erection and were considered erectile events. For each trace the following parameters were calculated:

1. percentage of rats displaying at least one ICP rise during the experiment
2. mean number of ICP rises per rat
3. duration of ICP rises
4. latency to first ICP rise

In addition to recording ICP, blood pressure (BP) was also recorded in each rat, as changes in blood pressure can impact on blood supply to the penile vasculature and so affect ICP responses in the anaesthetised rat (Giuliano et al, 1993). Thus, in these studies, the amplitude of each ICP rise was normalised according to the corresponding mean BP, termed ICPmax/BP ratio. If more than one ICP rise occurred during the test period then the ICPmax/BP ratio was averaged over the total number of ICP rises per rat, then per group. Additionally, the area under the curve of each ICP rise was calculated relative to the corresponding mean blood pressure (AUC/BP). If several ICP rises occurred then the SUM of AUC/BP of all ICP rises were calculated for each rat. The SUM AUC/BP gives an indication of overall erectile function. So the following additional measurements were also performed:

5. ICPmax/BP
6. SUM AUC/BP

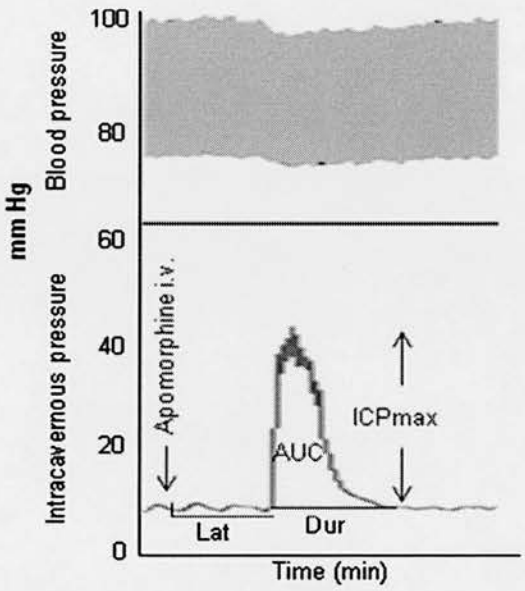


Figure 2.13: An example trace showing increased ICP after apomorphine injection (i.v.). Measured parameters for each ICP rise were as follows: latency to first response (Lat); duration of the response (Dur); area under the ICP curve (AUC); maximal ICP value reached during the ICP rise (ICPmax).

Figure 2.13 illustrates an example of a typical ICP and BP tracing after injection of a pro-erectile drug, such as apomorphine. As can be seen, the BP baseline remains relatively stable whilst, transient increases in the ICP baseline are observed after durg injection.

2.11 Analysis of oxytocin release in the lumbosacral spinal cord – sampling extracellular fluid

2.11.1 General principles

Initially microdialysis was used in this study to measure oxytocin release. The concept of microdialysis involves the use of a probe with a semi-permeable membrane at the tip that is inserted into tissue or fluid of interest and continually perfused with a cerebrospinal fluid substitute solution.

The probe membrane acts as an endogenous membrane whereby molecules diffuse across the membrane and down their concentration gradient.

In our studies, a microdialysis probe secured to a stereotaxic frame was inserted into the lumbosacral spinal cord of an anaesthetised male rat. For microdialysis, the probe was connected to a 10µl Hamilton syringe via polyethylene tubing and mounted on a perfusion pump set at an infusion rate of 10µl / min. The probe outlet tube was placed in a sterile eppendorf for sample collection.

We also used push-pull perfusion in our studies to extract extracellular fluid from the lumbosacral spinal cord. Conceptually, push-pull perfusion is similar to microdialysis, however, the probe does not contain a semi-permeable membrane and acts simply as a conduit for delivering perfusion fluid into extracellular space and removing extracellular fluid at the same rate. Thus, the limit on peptide uptake is much less restrictive than microdialysis and so allows the total amount neuropeptide in the extracellular fluid sample to be assayed. With push-pull perfusion, larger volumes of perfusate can be delivered and extracted from the area of interest. Thus, push-pull perfusion may allow extraction of samples with higher oxytocin concentrations that can be detected when assaying. Because push-pull perfusion extracts cells present in the extracellular space, the collected sample will contain those extracellular constituents in addition to oxytocin molecules, such as most amino acids and degradation enzymes. So it may be that the "purity" of collected sample is reduced when compared with microdialysis samples and the oxytocin present in the sample may be degraded by the enzymes also in the sample. However, by adding a preventative agent such as HCl should help to reduce the actions of the endogenous biodegradors.

Reagents

1. Artificial cerebrospinal fluid (ACSF) (pH 7.3-7.4): For 500ml volume, add 4.03g NaCl, 0.134g KCl, 0.39g $NaHCO_3$, 0.035g Na_2HPO_4, 0.09g $CaCl_2$, 0.120g $MgCl_2$ and 0.065g urea (all Sigma,

UK) to 500ml dH_2O. Osmolality was measured using an osmometer and adjusted to 300m osmol kg^{-1}/H_2O^{-1}.

2. 4% Paraformaldehyde in 0.1M PB
3. Toluidine blue: 10mg toluidine blue powder (Sigma, UK) and 100mg sodium borate (Sigma, UK) in 100ml dH_2O.
4. Acetic acid fixative: 10 ml formaldehyde (40%) (Sigma, UK) and 5ml glacial acetic acid (BDH, UK) in 85ml absolute ethanol.

2.11.2 Protocol for measuring oxytocin release

Rats were anaesthetised as described before and the dissection of the spinal cord and cannulation of the femoral vein were performed as previously described.

The rat was mounted in a stereotaxic frame. Stereotaxic ear bars were replaced with a 2ml syringe attached to a blunted 16G needle which would provide anchorage for the lumbosacral vertebae. The rat was placed on its ventral surface and each 16G needle was used to anchor each side of the L1 vertebra in the stereotaxic frame, this aided in exposing the L4-L6 region of spinal cord. Once fixed and secured, a plastic vial (approximately 5cm x 2cm x 2cm) was placed under the metal shaft of each blunted needle to provide additional support and to maintain the elevation of the spinal cord (Figure 2.14). This set up also helped to reduce movement of the lumbosacral spinal cord during breathing.

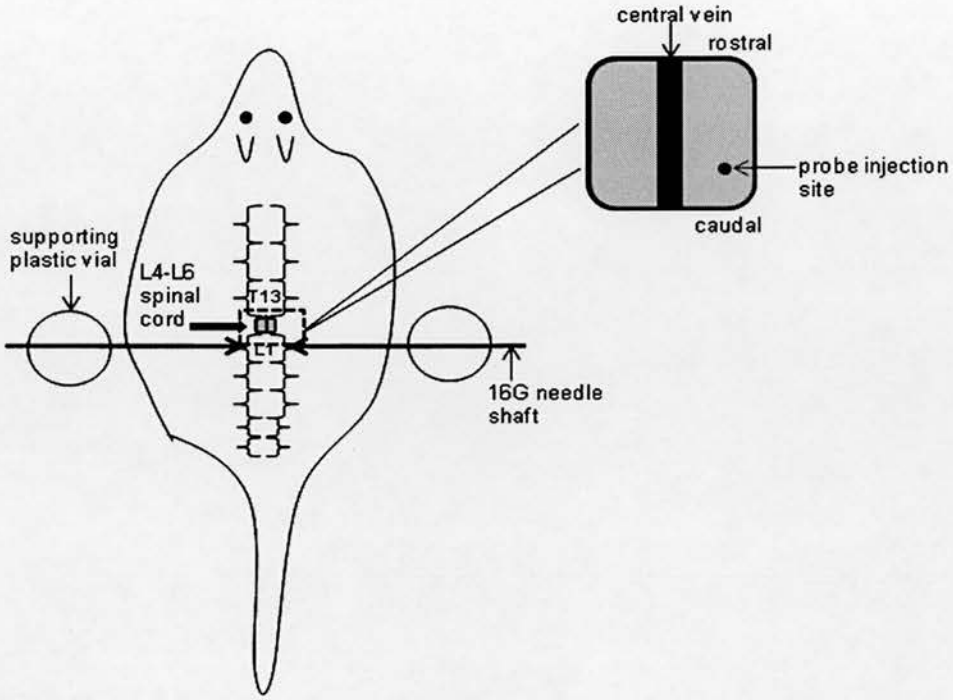


Figure 2.14: Technical set-up for push-pull perfusion. The L1 vertebra was anchored each side to a 16G needle. A supporting plastic vial was then placed under the metal shaft of each needle to help in elevating the spinal cord. Once elevated, the "window" exposing the L4-L6 spinal cord located in the junction between the T13 and L1 becomes widened allowing for more accurate insertions of the probe.

Additionally, a warmed paper blanket was wrapped around the rat and contained an opening round the thoracic cavity thus allowing the rat to breathe more freely and to reduce breathing-induced movement of the spinal cord. In order to avoid breathing difficulties and mucus accumulation in the throat, the head and neck of the rat was held at slight elevation. A microdialysis probe with the membrane removed was used for push-pull perfusion collection of extracellular fluid i.s. The push-pull probe was clamped in the probe holder of the stereotaxic frame and the inlet tube of the probe was connected to a tubing adaptor attached to fluorinated ethylene propylene tubing (FEP) (50 cm in length, diameter 0.12mm, CMA Microdilaysis, Linton Instrumentation, UK). The FEP tubing was then fitted to a 1ml Hamilton syringe filled with ACSF warmed to 37°C in the perfusion pump via another tubing adaptor.

The outlet tube of the probe was connected to FEP tubing (25cm in length, diameter 0.12mm) via a tubing adapter and then to larger FEP tubing (42cm in length, diameter 0.8mm, CMA microdialysis, Linton Instrumentation, UK) within the peristaltic pump (Figure 2.15). The probe was carefully lowered into the same lumbosacral site as previously described, once the probe was visibly adsorbed by the spinal cord tissue (approximately 1mm), the two pumps were started simultaneously to commence the equilibration period. The collecting tube was held continually in dry ice to freeze the sample as soon as possible. Samples were collected in dry ice in a sterile eppendorf containing 3% 0.2M HCl to help prevent the activity of endogenous degrading enzymes. Samples were then stored at -20° until processed by radioimmunoassay.

The injection site was carefully observed and monitored to ensure there was no fluid leakage and to avoid air bubbles entering the tubing. After an initial 30 min equilibration period, two baseline samples were collected and after injection of 250µg/kg apomorphine (or vehicle, saline + 0.1% ascorbic acid) at 90 min, three 30 min analyte samples were collected thereafter (Figure 2.16).

Since the dynamics of tissue and ACSF fluid are different, flow rates may change. Thus, the infusion pump (push) and peristaltic pump (pull) were calibrated twice before the start of each experiment; (1) prior to insertion of probe into the spinal cord and (2) after insertion of the probe into the spinal cord during the 30 min equilibration time. The probe was placed in either ACSF (1) or spinal cord tissue (2). The "push" pump was set at 10-11µl/min and the "pull" pump was set at a speed of 0.55 arbitary units. Measuring the weight of an eppendorf (determined gravi-metrically) before and after a 10 min sample collection period allowed an approximate calculation of the flow rate per min. In these experiments a flow rate of 10µl/min was used for a 30 min sample period sample to produce a sample volume

of 300µl.

After use, the probe and tubing were flushed with saline (30µl/min) and then distilled water (30µl/min) for 15 min each to dissolve any small blood clots surrounding the probe and to flush out any remaining ACSF within the tubing thus preventing microbial growth.

At the end of the experiment, the spinal cord segment containing the injection site and tissue approximately 2mm above and below the injection site was dissected out and placed in 4% paraformaldehyde for 48-72 hours before being cut on a cryostat at 30μm and stained with toludine blue to locate the site of injection as described previously.

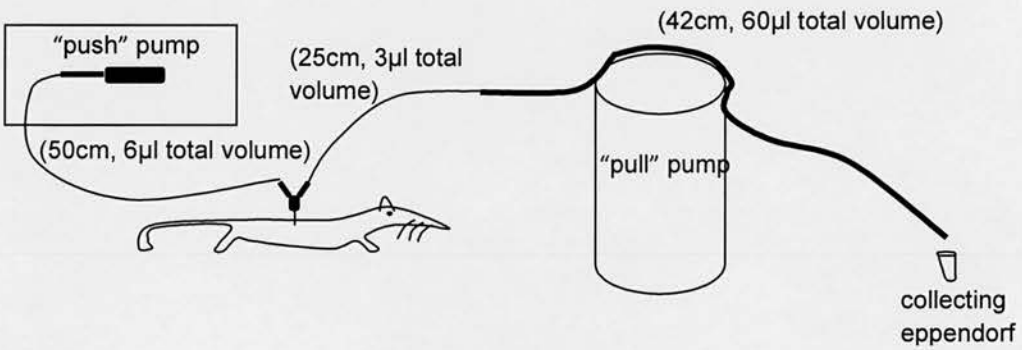
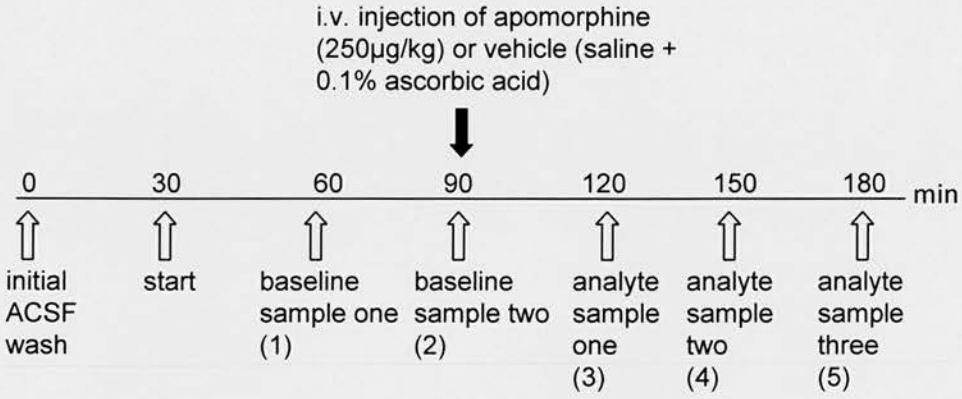


Figure 2.15: A push-pull probe, inserted into the lumbosacral spinal cord, is attached to a separate "push" and "pull" pump via FEP tubing. The area of interest is perfused with ACSF at set flow rates to generate a collecting sample of 300μl/min.

The total volume capacity for all tubing was 69μl of perfusion fluid. Thus at a flow rate of 10μl / min, it took approximately 7 min for the fluid to travel from the "push" pump to the "pull" pump and then to the collecting eppendorf. From the spinal cord to the collecting eppendorf it took approximately 6min and 18 sec (63μl), thus at the end of each 30 min sample a further 6 min 18 sec was allowed to collect the fluid from that sample travelling from the spinal cord thus avoiding contamination of samples (Figure 2.15). Thus, the time of i.v. injection of apomorphine or vehicle were calculated accordingly to ensure correlation with precise timing of sample collection.

Breathing frequency was recorded at various intervals over the experimental time period: 60, 85, 95, 185, 195 min. Breathing frequency was monitored by observing the number of inspirations over 1

Figure 2.16: Push-pull sample collection



Sample collections: after the collection of two 30 min baseline samples, drug or vehicle was injected at 90 min and three 30 min analyte samples were subsequently collected.

min at the time periods listed above. This was carried out because apomorphine can have adverse effects on respiration and can act as a permanent cardiovascular depressant. Also there was some slight movement around the probe injection site so it was important to know that any increase in oxytocin concentrations were due to the effect of apomorphine and not local tissue damage.

Oxytocin radioimmunoassay

Oxytocin radioimmunoassay was performed by Rainer Landgraf (Max Planck Institute of Psychiatry, Kraepelinstr.2, Munich) as described previously by Landgraf (1981). Briefly, perfusate samples were lyophilised and suspended in assay buffer. Samples were the redissolved in acidifed distilled water and then extracted using Chroprep Si60 (Merck, FRG). Sensitivity of the oxytocin radioimmunoassay was 0.3pg / sample and cross-reactivity levels with other related neuropeptides was less than 0.7%. Samples containing either perfusate or synthetic oxytocin were assayed at the same for validation.

2.12 General statistics

All data are presented as means ± SEM. The statistical tests applied for each experiment are detailed in each individual chapter, however, in general terms; the following statistical analyses were conducted. Analysis of all behavioural and neuronal activation data involved the use of either a Chi-squared test, One Way ANOVA (followed by an appropriate Post-Hoc test) or unpaired Student's t-test. For analysis of oxytocin release data, either an unpaired Student's t-test or One Way ANOVA for repeated measures was applied. P values <0.05 were considered statistically significant.

Chapter 3

The effect of dopamine D2-like ligands on penile erection-induced neuronal activation of hypothalamic oxytocin neurons

Chapter 3

3.1 The Effect of D2-like Receptor Ligands on Hypothalamic Oxytocin Neurons During Male Sexual Behaviour

Modulation of central dopaminergic function and their subsequent effects on sexual behaviour by relatively non-selective pharmacological tools such as the dopamine receptor agonist apomorphine and the dopamine receptor antagonists clozapine and haloperidol are well documented. (Guiliano and Allard for review, 2001; Brioni et al, 2004; Hsieh et al, 2004; Melis et al, 2005). D1-like receptors (D1, D5) have been implicated in the control of penile erection, where D1 receptor agonists can elicit spontaneous erections in conscious rats (D'Aquila et al, 2003) and D5 receptors seem to mediate the rewarding aspects of consummatory behaviour such as intromissions (Kudwa et al, 2005). However, it is the D2-like receptor (D2, D3, D4) studies that display the most convincing evidence and have an established role in the regulation of male copulatory function. The D2-like dopaminergic receptor agonists, Quinelorane (D2/D3 receptor agonist) and PD 168077 (selective D4 receptor agonist) have long been shown to have facilitatory effects on penile erection (Foreman and hall, 1987; Eaton et al, 1991; Bazzett et al, 1991; Doherty and Wisler, 1994; Melis et al, 2005; Melis et al, 2006). Likewise, the D2-like dopaminergic receptor antagonists, L-741,626 (D2 preferring receptor antagonist), nafadotride (D3 preferring receptor antagonist) and L-745,870 (D4 preferring receptor antagonist) are able to reduce the occurrence of various copulatory parameters in male rats such as ejaculation, penile erection, mounting and intromitting behaviour (Clement et al, 2007; Melis et al, 2005; Melis et al, 2006; Millan et al, 2000; Stafford and Coote, 2006; Succu et al, 2007). The above dopamine receptor ligands show comparatively greater selectively for the D2-like receptors than the conventional pharmacological agents previously described.

3.1.1 Dopamine-oxytocin interactions

The precise D2-like dopamine receptor(s) and neural pathways activated by dopaminergic agents during erectile function remain to be elucidated. Central serotonergic and cholinergic pathways have been proposed to partly mediate the pro-erectile effects of dopamine agonists (Maeda et al, 1994;

Stancampiano et al, 1994). However, central oxytocinergic pathways originating in the PVN, have received greater attention, primarily due to fact that their central projections terminate in areas known to be actively involved in sexual behaviour, namely, MPOA, VTA, amygdala and the spinal cord (Condes-Lara et al, 2007; Kirchgessner et al, 1988; Longueville et al, 1999; Roeling et al, 1993). In vitro studies using hypothalamic explants, have shown that dopamine and the antagonist, haloperidol, can increase and decrease oxytocin release respectively. Additionally, *in-vivo* studies have shown that intra-PVN, intracerebroventricular (i.c.v.) or peripheral administration of dopamine or the non-selective dopaminergic agonist, apomorphine, increases release of oxytocin into the blood and hippocampus in rats and monkeys, an effect which occurs concomitantly with the expression of penile erection (Argiolas, 1999; Cameron et al, 1992; Melis et al, 1990, 1992). However, it is not known if dopamine acting in the MPN, SON or PVN can influence oxytocin neuron activity (and perhaps oxytocin release) within the hypothalamus and if such an effect occurs in parallel with sexual behaviour. It has recently been shown that during copulation there is increased oxytocin release within the PVN (Waldherr and Neumann, 2007). Because we know that dopamine and oxytocin action in the PVN can produce similar facilitatory effects on sexual behaviour, it seems plausible to assume that dopamine may enhance oxytocin release in the PVN during the expression of copulatory behaviour. Additionally, electrical stimulation of the PVN increases release of oxytocin in the nucleus tractus solitarius (NTS) and the dorsal motor nucleus of the vagus nerve (DMV) (Landgraf et al, 1990) implicating PVN oxytocin fibres in the mediation of autonomic functions which partly mediate penile erecion. Two other oxytocin-containing brain nuclei that have been implicated in sexual behaviour are the MPN and SON. However, the involvement of these oxytocin populations in the expression of penile erection after dopamine agonist administration is not known.

3.1.2 Neuronal activation

Fos immunocytochemistry has proved to be an extremely powerful investigative tool when trying to establish potential brain neural networks subserving behaviours, particularly sexual behaviour (Pfaus and Heeb, 1997; Robertson et al, 1991). It allows mapping of functional neural networks (specific localisation of anatomical structures expressing Fos due to a physiologic or behavioural stimuli) and provides high cellular resolution. Fos (protein product of *c-fos* gene) expression is used as a marker

for neuronal activation and can often be combined immunocytochemically to identify a range of peptidergic neuronal phenotypes such as vasopressin and oxytocin. During intromission and ejaculation, supraoptic oxytocin neurons are activated (Caquineau et al, 2006; Pattij et al, 2005) and oxytocin-induced penile erection increases Fos expression in oxytocin neurons in the paraventicular parvocellular (pPVN) oxytocin neurons (Kita et al, 2006). Additionally, masculine sexual behaviour increases oxytocin neuronal activation in subregion-specific divisions of the pPVN (Witt and Insel, 1994). Regarding the MPN (part of MPOA), copulation-induced Fos expression has been shown (Lumley and Hull, Balthazart and Ball, 2007); however, the neuronal phenotype expressing the Fos protein is not known. Copulation-induced Fos expression in oxytocin neurons has not previously been investigated in the MPN. Furthermore, regarding the MPN, SON and PVN, the effect of D2-like dopaminergic ligands on sexual behaviour-induced Fos expression in these three hypothalamic nuclei is unknown.

The PVN is one brain area in particular that is highly sensitive to the pro-erectile effects of apomorphine so it is believed that oxytocin neurons in the PVN mediate the pro-erectile effects of apomorphine. However, it is not known if oxytocin neurons in the MPN and SON also partly mediate the pro-erectile effects of dopamine. Thus, the aim of this study was to replicate the facilitatory and inhibitory effects of dopamine agonists and antagonists on male sexual behaviour as previously reported and to investigate the effects of these pharmacological agents on hypothalamic oxytocin neurons (in the MPN, SON and PVN). Therefore, our hypothesis was that D2-like agonists and antagonists facilitate and reduce the incidence of penile erection in the conscious male rat, respectively and do so via the activation and attenuation of oxytocin neuronal activity, respectively, in the MPN, SON and PVN. To test this hypothesis, the study was comprised of two parts; (1) to investigate oxytocin neuronal activation associated with D2-like agonist-induced penile erection and (2) to examine the effect of intromission-induced oxytocin neuronal activation after dopamine receptor blockade.

Chapter 3

3.2 Methods

3.2.1 Effects of D2-like agonists on penile erection and the activation of oxytocin neurons

3.2.1.1 Animals

Naïve Sprague-Dawley male rats (200-250g) were used and maintained throughout on a 12:12h light dark cycle (lights on from 07:00am to 7:00pm). Experiments were conducted between 11am and 2pm.

3.2.1.2 Drugs

D2/D3 agonist, Quinelorane (EC_{50} values: 0.7, 1.7, 7 nM for the D2, D3 and D4) or selective D4 agonist, PD 168077 (EC_{50} values: 5.6, >10,000, >10,000 for D4, D3, D2) (both Sigma, UK) were dissolved in saline, diluted with distilled water and administered i.c.v. in a 2µl volume over 2 min. A dose of 1µg Quinelorane was administered as previously done by Bitran and colleagues (1989) for erectile observations. At the time of this study, PD 168077 had not been injected i.c.v. according to the literature. It had however, been injected unilaterally into the PVN (1µg) (Melis et al, 2005), thus an approximate estimation of 4.5µg was injected i.c.v. Interestingly, in a later published study, where a dose-response curve was constructed, 5µg (i.c.v.) of PD168077 was shown to be the submaximal dose for generating erection episodes in conscious male rats (Melis et al, 2006).

3.2.1.3 Behavioural observations

After i.c.v. cannulation, rats were handled daily (as explained in General Methods). Two days after surgery, rats were housed individually in Plexiglas cages (30cm x 30cm x 30cm) during observation and recording of behaviour. Rats were injected with Quinelorane (1µg) (n=8), PD168077 (4.5µg) (n=10) or vehicle (saline, 2µl) (n=8) via an i.c.v. injection cannula over 2 min. The injection cannula was kept in place for a further 30 sec to allow diffusion and prevent backflow of the drug in to the injection cannula. After drug injection, the number of stretching, yawning and penile erection episodes were counted for 60 min. Penile erection was scored when the penis emerged from the

penile sheath, accompanied by penile grooming and hip flexion. Additionally, any apparent changes in motor function were also noted during the observation period.

3.2.1.4 Perfuse fixation and immunocytochemistry

Ninety min after drug administration, rats were terminally anaesthetised with sodium pentobarbitone (i.p.) and perfused transcardially with 200 ml of 0.9% heparinised saline followed by 300 ml of 4% paraformaldehyde in 0.1M PB (pH 7.35-7.4) (as described in General Methods, section 2.6). Brains were then removed and placed in post-fix solution (50% 4% paraformaldehyde and 50% 30% sucrose) for 24 hr followed by immersion in 30% sucrose solution for 48-72 hr. Brain sections containing the MPN, SON and PVN were sliced into 48µm coronal sections using a freezing microtome.

Fos and combined Fos and oxytocin immunocytochemistry was carried out using the ABC (avidin-biotin peroxidase complex) method as explained in General Methods sections 2.7.1 - 2.7.3.

3.2.1.5 Immunocytochemistry analysis

Fos

Using a light microscope (x25 magnification) and on coded slides, Fos-positive cells were counted as those with black nuclear staining in the MPN, SON and PVN. For the MPN and SON, approximately 2-4 sections per rat were counted and area per section profile calculated. For the PVN, Fos-positive cells were quantified in 4 sections per rat in the following PVN subdivisions; anterior parvocellular, dorsal parvocellular, medial parvocellular, lateral parvocellular and posterior magnocellular. The average per profile section was calculated and then summed together to give a value of the PVN as a whole. All values were averaged per rat and then per group.

Fos and oxytocin co-localisation

For quantification of Fos and oxytocin cells, the counts were performed in each of the brain areas as described above. In addition to Fos only counts, the total number of oxytocin cells and co-localised oxyocin cells with Fos protein were quantified separately in the each of the three brain nuclei. The

number of Fos-positive oxyocin cells were expressed as a percentage of Fos and oxytocin-expressing cells divided by the total number of oxytocin cells.

3.2.1.6 Statistics

The incidence of penile erection was defined as the percentage of rats displaying one or more erections during the 60 min observation period. Statistical evaluation was determined by Chi-squared test with Yates correction where $P<0.05$ was considered significant. The mean number of penile erections were analysed between groups using One Way ANOVA when the data was normally distributed. When the data was not normally distributed, a One Way ANOVA on Ranks followed by Dunn's multiple comparison test or Mann-Whitney rank sum test was performed. P values <0.05 were considered significant. Differences in the numbers of Fos-positive neurons or the percentage of Fos-positive oxytocin cells were assessed for statistical significance across treatment groups by One Way ANOVA and where necessary, post hoc Dunn's or Student-Newman-Keuls pairwise comparison tests were applied. When the data was not normally distributed a Kruskal-Wallis One Way ANOVA on Ranks test was perfomed. As above P values <0.05 were considered significant.

3.2.2 Effects of D2-like antagonists on intromission and the activation of oxytocin neurons

3.2.2.1 Animals

Male and female Sprague-Dawley rats (200-250g) were used and placed under reverse light cycle on a 12:12h dark light cycle (lights on 7:00pm to 07:00am). Experiments were conducted between 11am and 2pm when female rats were sexually receptive and males were at their most sexually rigorous. Sexually-experienced male rats were used to ensure they were capable of copulation on the day of the experiment. On experiment day, females were screened by placing them with spare virgin males and only those females who were highly receptive were used in the study.

3.2.2.2 Drugs

The D2 antagonist, L-741, 626 (D2A), D3 antagonist, nafadotride (D3A) and D4 antagonist, L-745, 870 (D4A) were dissolved in DMSO (for L-741,626) and saline (for nafadotride and L-745,870) and then diluted with saline. Each drug was injected i.c.v. in a 2µl volume over 2 min. Ki values are shown in Table 3.1.

	D2 receptor	D3 receptor	D4 receptor
D2A	2.4	100	220
D3A	5	0.52	269
D4A	960	2300	0.51

Table 3.1: Ki values (nM) for the D2A, D3A and D4A.

3.2.2.3 Behavioural observations

After i.c.v. cannulation rats were placed into individual cages and handled daily (as explained in General Methods). Two days after surgery and on experiment day, each male rat was placed into a transparent cage of a receptive female to allow observation and recording of behavior. Males were placed in to the receptive female's cage to reduce the levels of stress (which would be increased if placed in a novel environment i.e. an adult male's cage) endured by the female rat and thus make the female more likely to mate with the male rat. This experimental protocol for sexual behavior studies has been published before from our lab (Caquineau et al, 2006). Males were originally placed into five different drug groups: vehicle (DMSO, n=14, control for L-741,626); D2 receptor antagonist (L-741,626, n=9, 1µg and n=12, 10µg); saline (n=12, control for nafadotride and L-745,870); D3 receptor antagonist (nafadotride, n=12, 5µg) and D4 receptor antagonist (L-745,870, n=18, 7µg). The males were then randomly assigned to two separate groups: those placed alone in a novel environment with no female or female cues (ALONE) and those placed with a receptive female and allowed to intromit (I). The experiment was carried out on three consecutive weeks. It was soon apparent that during the test period, some rats (vehicle- and drug-treated rats) displayed mounting but not intromitting behavior. Thus, those rats that did not intromit in this study, comprised a non-

intromitting (NI) group. Thus, for each drug treatment and corresponding vehicle group, there were the following groups; ALONE, NI and I.

Drug injection was given (as described previously) 15 min prior to the male being placed with the receptive female. Once placed in the cage with the female the following copulatory parameters were recorded for 15 min; mount and intromission latency (ML and IL) and mount and intromission frequency (MF and IF).

3.2.2.4 Perfuse fixation and immunocytochemistry

Perfusions, Fos and combined Fos and oxytocin immunocytochemistry and analysis were performed as described in General Methods (and *section 3.2.2.3)*.

3.2.2.5 Statistics

The incidence of mounting and intromitting was defined as the percentage of rats displaying one or more mounts and intromissions during a 15 min observation period. Statistical evaluation was determined by Chi-squared test with Yates correction where $P<0.05$ was considered significant. The mean number of mounts and intromission and the frequency of mounts and intromissions were analysed between groups using One Way ANOVA when the data was normally distributed. When the data was not normally distributed, a One Way ANOVA on Ranks followed by Dunn's multiple comparison test or Mann-Whitney rank sum test was performed. P values <0.05 were considered significant. Differences in the numbers of Fos-positive neurons or the percentage of Fos-positive oxytocin cells were assessed for statistical significance across treatment groups by One Way ANOVA and where necessary, post hoc Dunn's or Student-Newman-Keuls pairwise comparison tests were applied. When the data was not normally distributed a Kruskal-Wallis One Way ANOVA on Ranks test was perfomed. As above P values <0.05 were considered significant.

Chapter 3

3.3 Results

3.3.1 Effects of dopamine agonists on penile erection and neuronal activation in the MPN, SON and PVN.

3.3.1.1. Effects of D2-like agonists on general behaviour

Rats receiving Quinelorane and PD168077 at the doses used did not exhibit any extrapyramidal side effects such as those often associated with administration of dopaminergic agonists which may have otherwise impaired their ability to achieve penile erection.

3.3.1.2. Effects of D2-like agonists on penile erection

Quinelorane (1µg) or PD168077 (4.5µg) given to sexually-experienced male rats (i.c.v), elicited significant increases in the incidence (expressed as a percentage) of penile erection (Figure 3.1A) (Chi-squared test with Yates correction, $P<0.005$; $P<0.05$ vs vehicle) compared to vehicle-treated rats, which did not exhibit any penile erection episodes. Interestingly, 2/7, 6/8 and 2/10 rats exhibited yawning behaviour (not shown) and 3/7, 6/8 and 4/10 exhibited stretching behaviour (not shown) after vehicle, Quinelorane and PD168077, respectively. Quinelorane but not PD168077 also significantly increased the mean number of erections per rat (Figure 3.1B) (One-way ANOVA on Ranks followed by Dunn's multiple comparison test, $P<0.05$).

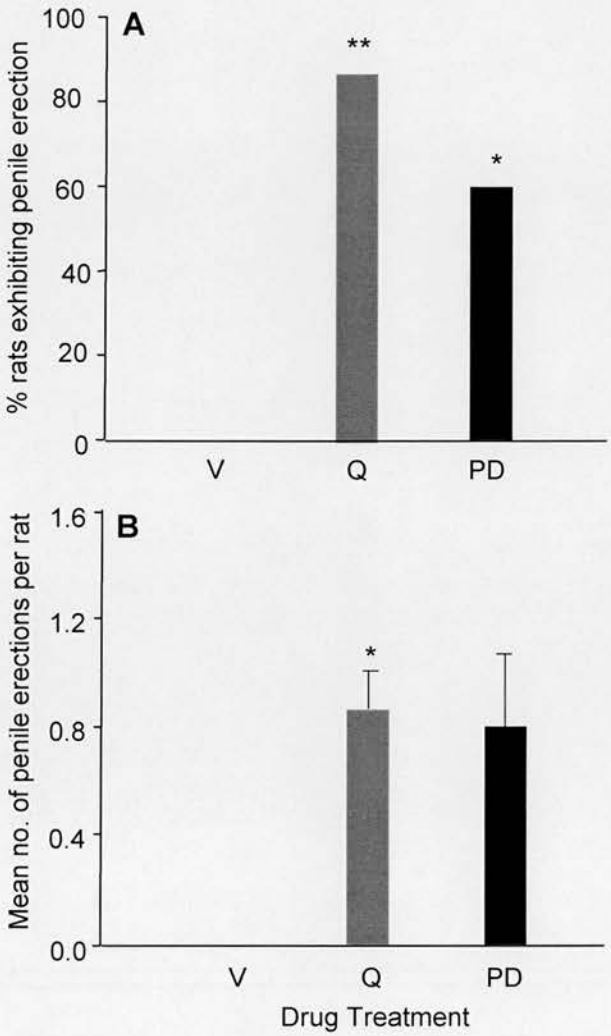
3.3.1.3. Effects of D2-like agonists on Fos expression

MPN

Photomicrographs in Figure 3.2A illustrate Fos expression in the MPN after i.c.v. administration of Vehicle, Quinelorane and PD168077. When quantified (Figure 3.2B), neither Quinelorane or PD168077 significantly increased the number of Fos-positive nuclei in the MPN compared to Vehicle (One-Way ANOVA).

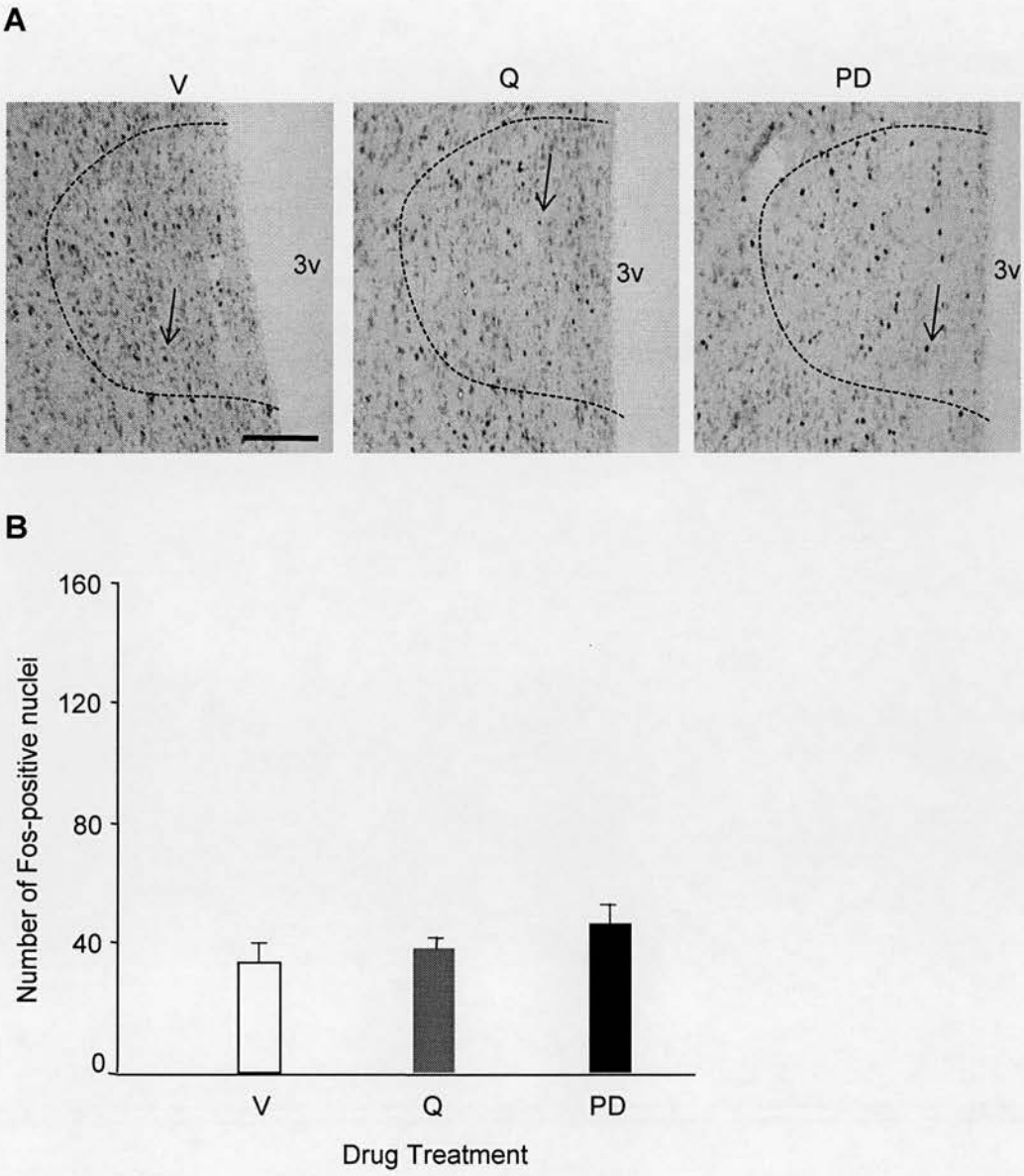
SON

Figure 3.1: Effects of Quinelorane and PD 168077 on penile erection in the conscious rat.



Effect of Quinelorane (Q, n=8, 1µg) and PD-168077 (PD, n=10, 4.5µg) on the incidence (%) of penile erection (A) and mean number of penile erections per rat (B). Male rats were observed for 60 min to quantify penile erection episodes. Values are the group means ± SEM. Q and PD significantly increased the % of rats exhibiting penile erection compared to V (Chi-sqaure test with Yates correction , *P<0.05; **P=0.005 vs V). Q but not PD significantly increased the mean number of penile erections per rat compared to V (One-way ANOVA on Ranks followed by Dunn's multiple comparison test, *P<0.05).

Figure 3.2: Effects of Quinelorane and PD168077 on Fos expression in the MPN



Effects of Vehicle (V, n=7, 2µl), Quinelorane (Q, n=8, 1µg) and PD168077 (PD, n=10, 4.5µg) on Fos expression in the MPN. V, Q and PD-induced Fos expression in the MPN are illustrated in the photomicrographs in 3.2A. The dotted line shows the area comprising the MPN. Values are the group means ± SEM. In Figure 3.2B, neither Q or PD had any significant effect on Fos expression in the MPN (One Way ANOVA) compared to V. Arrows indicate Fos-positive nuclei. Scale bars represent 100µm. 3v=third ventricle.

Photomicrographs in Figure 3.3A show Fos expression in the SON after i.c.v administration of Vehicle, Quinelorane and PD168077. Upon quantification (Figure 3.3B), neither Quinelorane or PD168077 significantly increased the number of Fos-positive nuclei in the SON compared to Vehicle (Kruskal-Wallis One Way ANOVA on Ranks).

PVN

Photomicrographs in Figure 3.4A show Fos expression in the PVN after i.c.v administration of Vehicle, Quinelorane and PD168077. Quinelorane but not PD168077 induced significant Fos expression in the parvocellular PVN (pPVN) (One-Way ANOVA followed by Dunnett's multiple comparison test, *P=0.05, Figure 3.4B) but not the magnocellular PVN (mPVN) compared to the corresponding Vehicle.

PVN subdivisions

Photomicrographs in Figure 3.5A illustrate Fos expression in the medial parvocellular subdivision of the PVN after i.c.v. administration of Vehicle or Quinelorane. Quinelorane but not PD168077 significantly increased Fos expression in the medial parvocellular subregion of the PVN One-Way ANOVA followed by Dunnett's multiple comparison test, *P<0.05) compared to Vehicle (Figure 3.5B). No effects were seen in any of the other PVN subdivisions after drug/vehicle administration.

3.3.1.4. Effects of D2-like agonists on Fos expression in oxytocin neurons

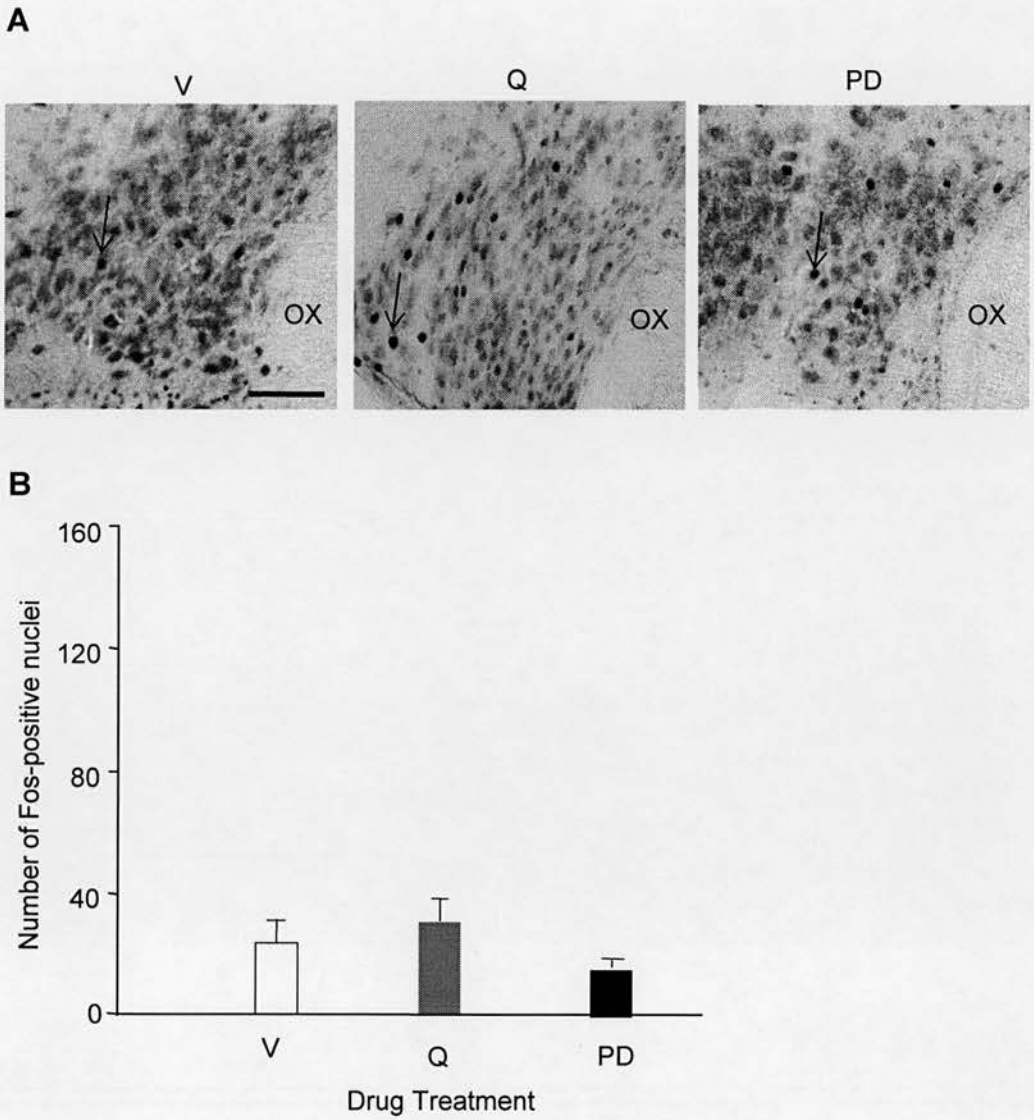
MPN

Photomicrographs in Figure 3.6A show Fos expression in oxytocin neurons in the MPN after i.c.v. administration of Vehicle, Quinelorane and PD168077. Quinelorane and PD168077 did not have any significant effect on Fos expression in oxytocin neurons in the MPN (Kruskal-Wallis One Way ANOVA on Ranks) compared to Vehicle (Figure 3.6B).

SON

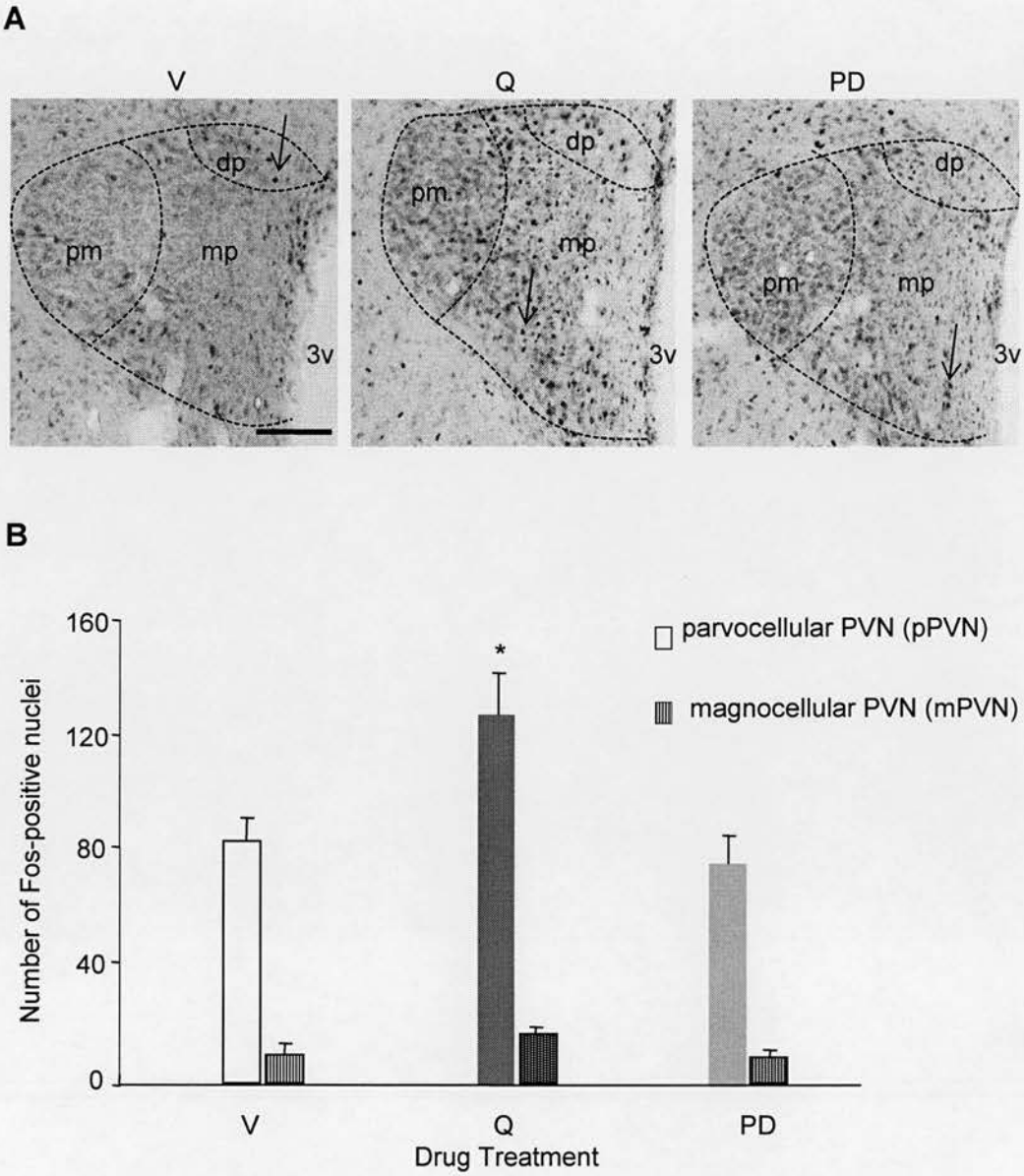
Photomicrographs in Figure 3.7A show Fos expression in oxytocin neurons in the SON. after i.c.v. administration of Vehicle, Quinelorane and PD168077. Quinelorane and PD168077 did not have any

Figure 3.3: Effects of Quinelorane and PD168077 on Fos expression in the SON



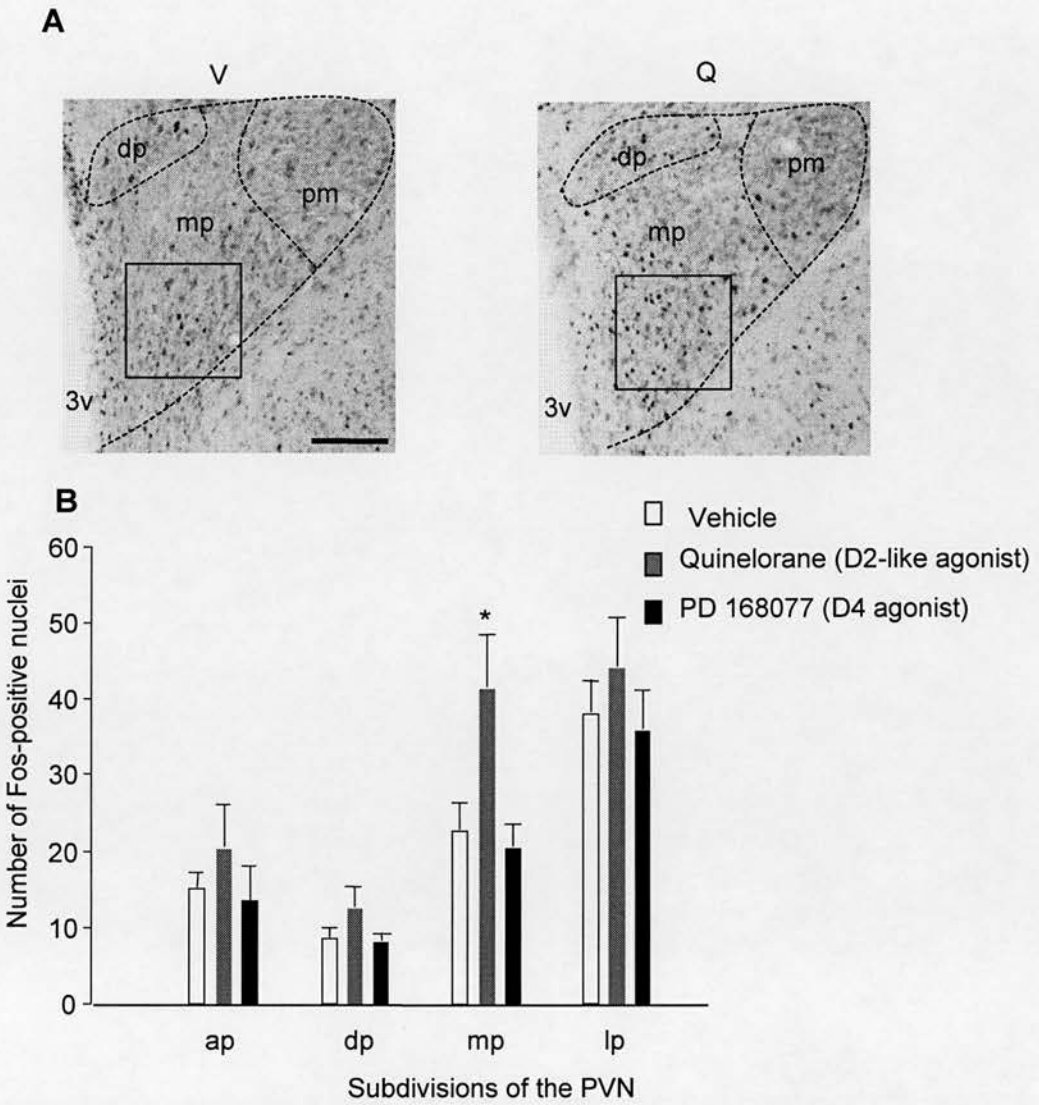
Effects of Vehicle (V, n=7, 2µl), Quinelorane (Q, n=8, 1µg) and PD168077 (PD, n=10, 4.5µg) on Fos expression in the SON. V, Q and PD-induced Fos expression in the SON are illustrated in the photomicrographs in Figure 3.3A. Values are the group means ± SEM. As seen in Figure 3.3B, neither Q or PD had any significant effect on Fos expression in the SON (Kruskal-Wallis One Way ANOVA on Ranks) compared to V. Arrows indicate Fos-positive nuclei. Scale bars represent 50µm. OX=optic chiasm.

Figure 3.4: Effects of Quinelorane and PD168077 on Fos expression in the PVN



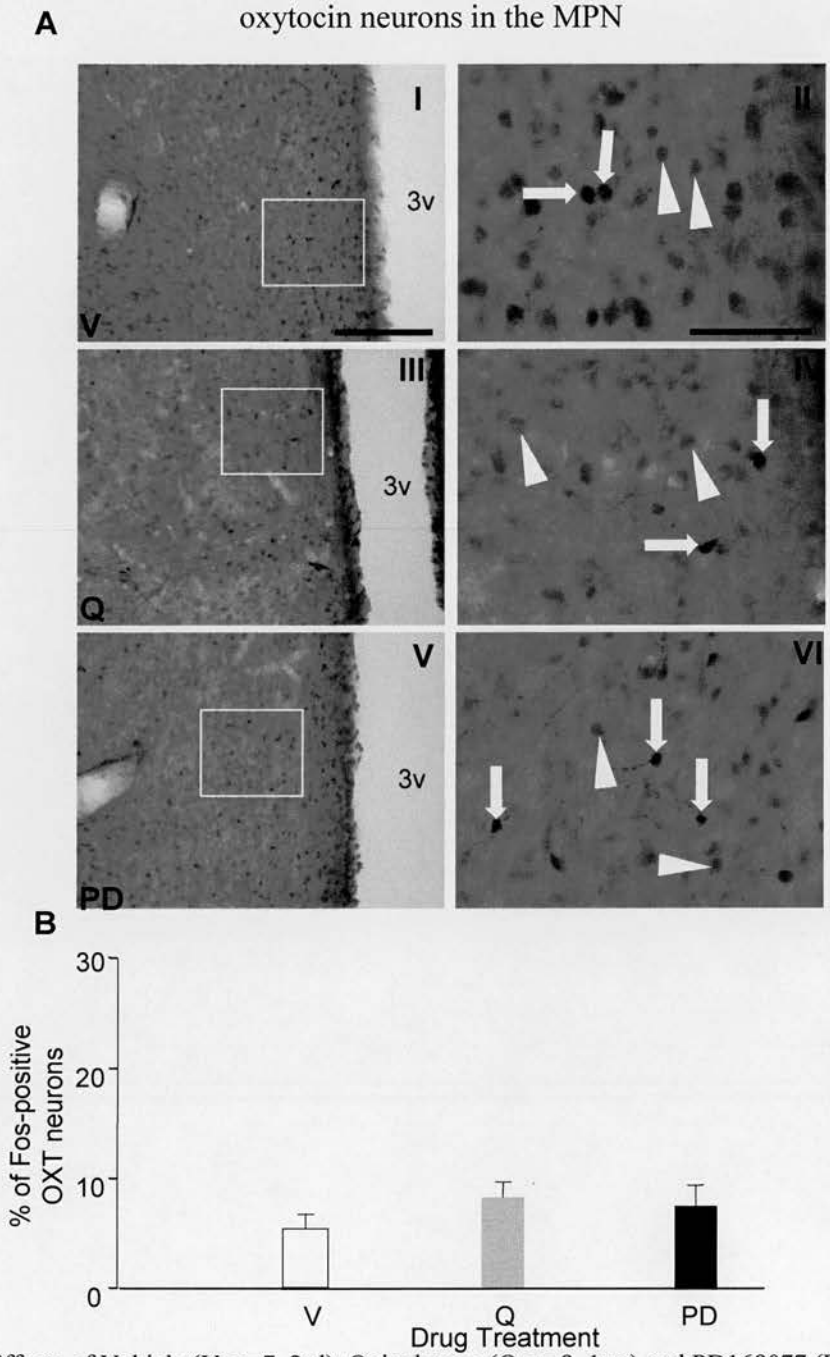
Effects of Vehicle (V, n=7, 2µl), Quinelorane (Q, n=8, 1µg) and PD168077 (PD, n=10, 4.5µg) on Fos expression in the PVN. V, Q and PD-induced Fos expression in the PVN are illustrated in the photomicrographs in Figure 4.4A. Values are the group means ± SEM. As seen in Figure 4.4B Q but not PD significantly increased Fos expression in the pPVN (One-way ANOVA followed by Dunnett's multiple comparison test, *P=0.05) compared to V. No differences in Fos expression were observed between Q, PD and V in the mPVN. Arrows indicate Fos-positive nuclei. Scale bars represent 100µm. 3v=third ventricle; dp=dorsal parvocellular; mp=medial parvocellular; pm=posterior magnocellular.

Figure 3.5: Effects of Quinelorane and PD168077 on Fos expression in the subdivisions of the PVN



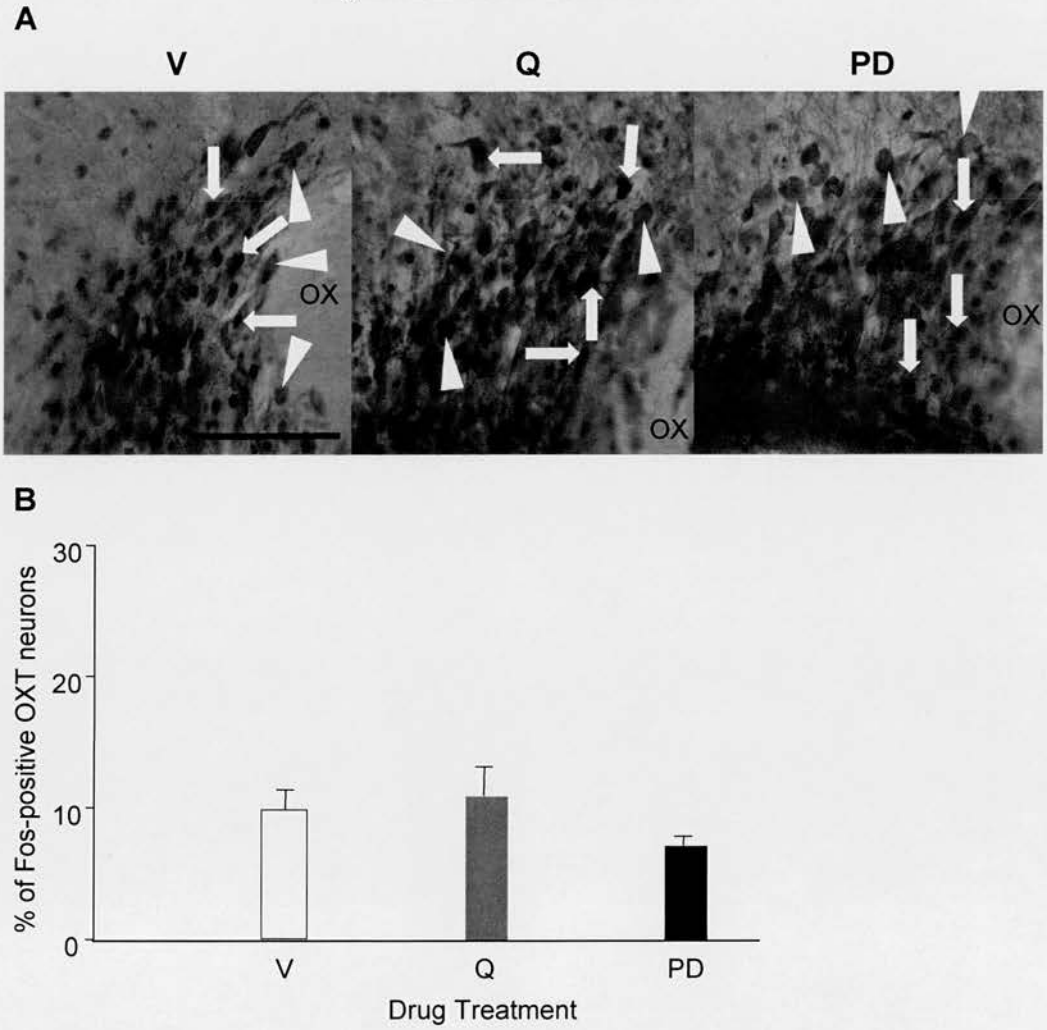
Effects of Vehicle (V, n=7, 2µl) and Quinelorane (Q, n=8, 1µg) on Fos expression in the subdivisions of the PVN. V and Q-induced Fos expression in the medial parvocelllular (mp) subregion of the PVN are illustrated in the photomicrographs in Figure 3.5A. Values are the group means ± SEM. As seen in Figure 5.3B Q but not PD significantly increased Fos expression in the the mp subdivision of the PVN (One-way ANOVA followed by Dunnett's multiple comparison test, *P<0.05) compared to V. No differences in Fos expression were observed between treatment groups in any of the other PVN subdivisions. Scale bars represent 100µm. 3v=third ventricle; ap=anterior parvocellular; dp=dorsal parvocellular; mp=medial parvocellular; lp=lateral parvocellular; pm=posterior magnocellular.

Figure 3.6: Effects of Quinelorane and PD168077 on Fos expression in oxytocin neurons in the MPN



Effects of Vehicle (V, n=7, 2µl), Quinelorane (Q, n=8, 1µg) and PD168077 (PD, n=10, 4.5µg) on Fos expression in oxytocin neurons in the MPN. V, Q and PD-induced Fos expression in the MPN are illustrated in the photomicrographs in Figure 3.6A. The dotted line shows the area comprising the MPN. Values are the group means ± SEM. As seen in Figure 3.6B, Neither Q or PD had any significant effect on Fos expression in oxytocin neurons in the MPN (Kruskal-Wallis One Way ANOVA on Ranks) compared to V. Scale bars represent 100µm and 50µm in I, 111, V and II, IV, VI respectively. Arrows show a Fos-positive oxytocin cell and arrowheads illustrate a Fos-negative oxytocin cell. 3v=third ventricle.

Figure 3.7: Effects of Quinelorane and PD168077 on Fos expression in oxytocin neurons in the SON



Effects of Vehicle (V, n=7, 2µl), Quinelorane (Q, n=8, 1µg) and PD168077 (PD, n=10, 4.5µg) on Fos expression in the SON. V, Q and PD-induced Fos expression in the SON are illustrated in the photomicrographs in Figure 3.7A. Values are the group means ± SEM. As seen in Figure 3.7B, neither Q or PD had any significant effect on Fos expression in oxytocin neurons in the SON (One Way ANOVA) compared to V. Scale bars represent 100µm. Arrows shows Fos-positive oxytocin cells and arrowheads illustrate Fos-negative oxytocin cells. OX=optic chiasm.

effect on Fos expression in oxytocin neurons in the SON (One Way ANOVA) compared to Vehicle (Figure 3.7B).

PVN

Photomicrographs in Figure 3.8A and 3.9A show Fos expression in oxytocin neurons in the PVN after i.c.v. administration of Vehicle, Quinelorane and PD168077. As seen with the Fos only results, Quinelorane but not PD168077 significantly increased the percentage of Fos-positive oxytocin cells expressed in the pPVN (One-way ANOVA followed by Dunnett's multiple comparison test, P=0.05) but had no effect in the mPVN compared to Vehicle (Figure 3.8B).

PVN subdivisions

As seen in Figure 3.9A and 3.9B, Quinelorane but not PD168077 significantly increased the percentage of Fos-positive oxytocin cells expressed in the medial parvocellular (mp) subdivision of the PVN (One-way ANOVA followed by Dunnett's multiple comparison test, P=0.001) compared to Vehicle. No differences between the drug groups were seen in any of the other PVN subregions.

3.3.2 Effects of D2-like antagonists on intromission and neuronal activation in the MPN, SON and PVN.

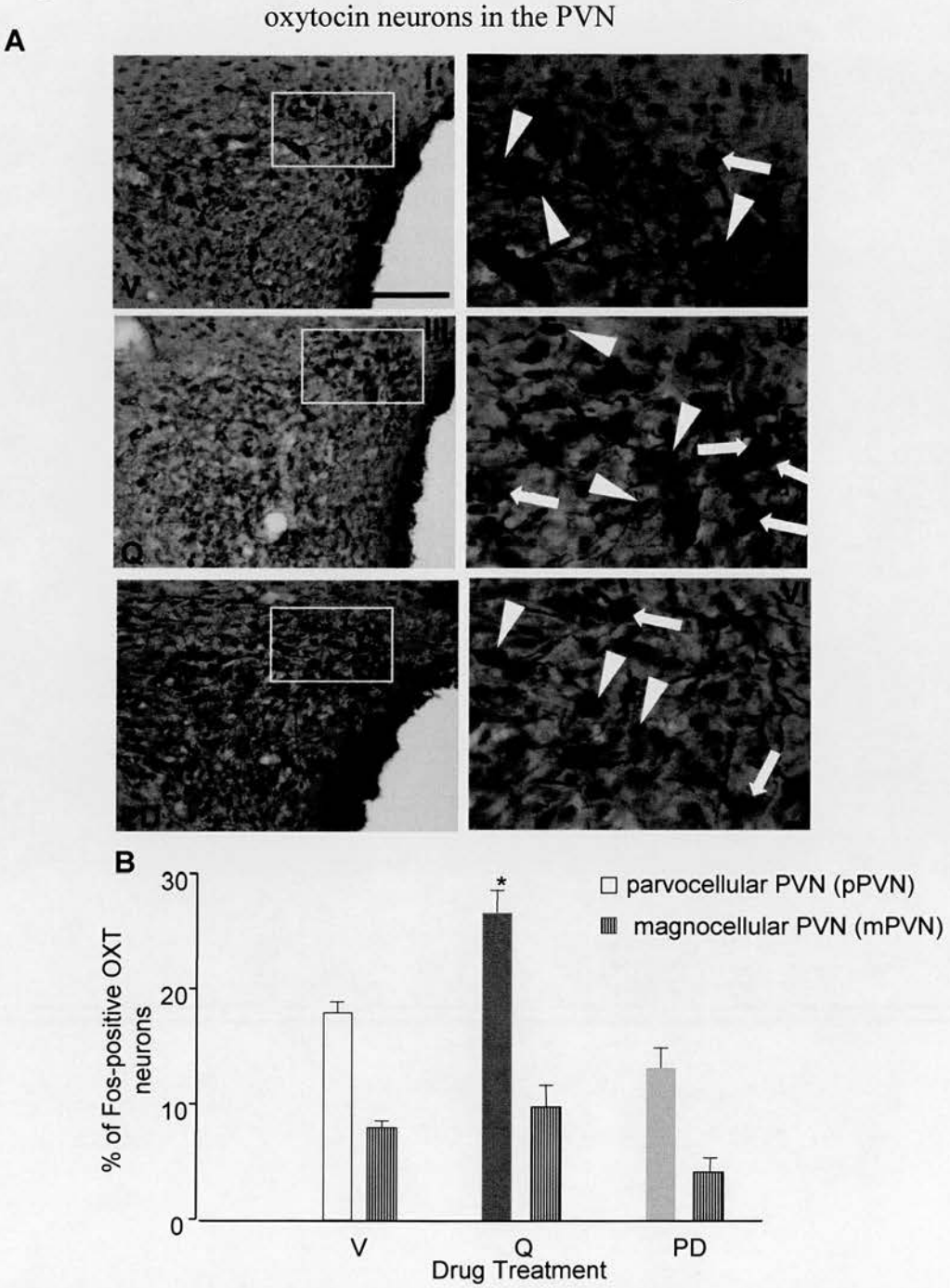
3.3.2.1 Effects of D2-like antagonists on general behaviour

At the doses used, no apparent impairments in motricity or gait, often associated with administering high concentrations of dopamine antagonists, were observed in rats treated with D2A, D3A and D4A (data not shown).

3.3.2.2 Effects of D2-like antagonists on the incidence of intromission

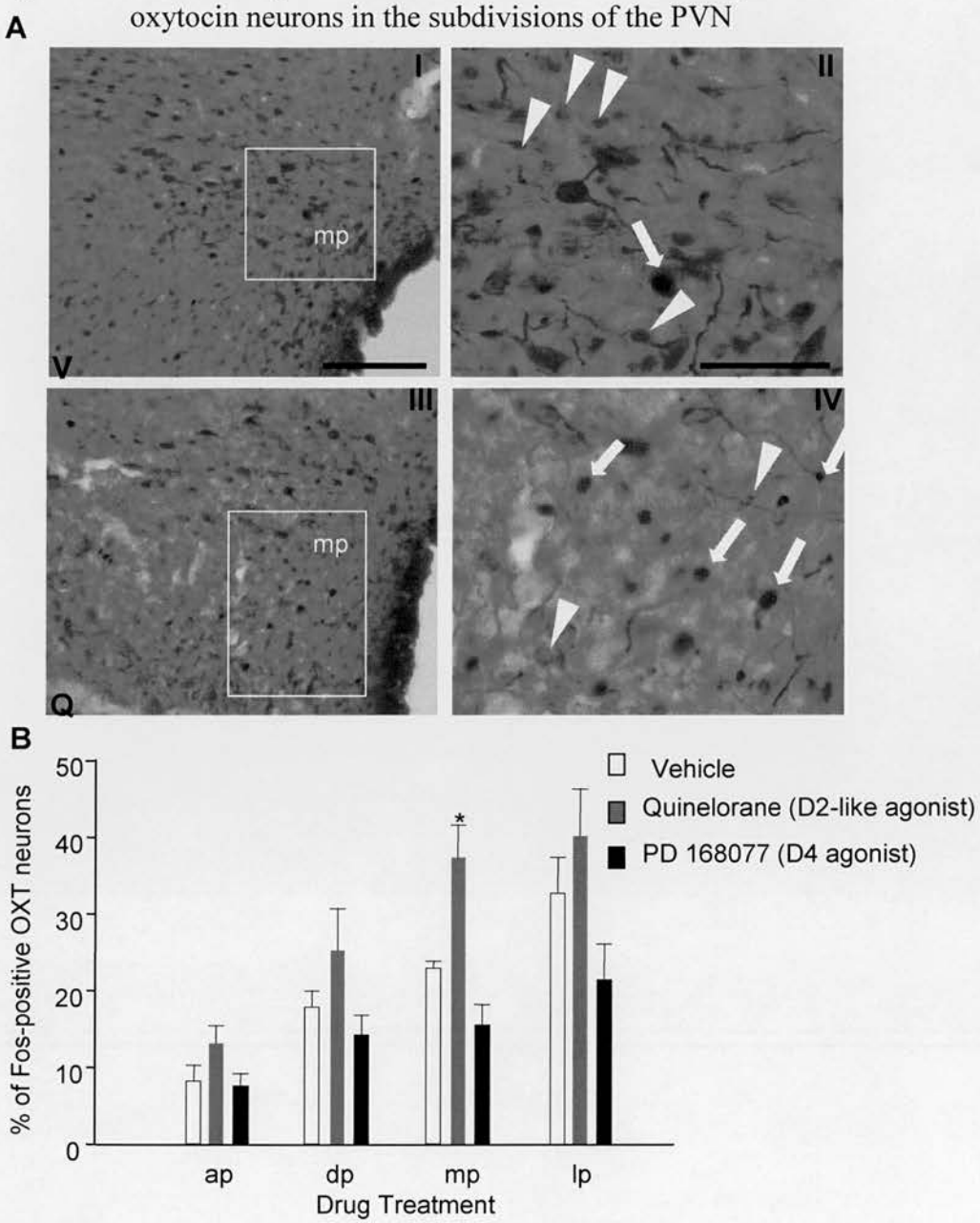
Sexually-experienced male rats treated with D2A (1μg or 10μg), D3A (5μg), D4A (7μg) or the corresponding vehicle (DMSO or saline) (all i.c.v.) displayed mounting and intromitting behaviour during the 15 min test period. Such behaviour was shown as the incidence (expressed as the percentage) of mounting and intromitting. Only those rats displaying one or more mounts and only those displaying one or more intromissions were included in statistical analysis. Those rats receiving the D2A (1μg or 10μg)

Figure 3.8: Effects of Quinelorane and PD168077 on Fos expression in oxytocin neurons in the PVN



Effects of Vehicle (V, n=7, 2µl), Quinelorane (Q, n=8, 1µg) and PD168077 (PD, n=10, 4.5µg) on Fos expression in the PVN. V, Q and PD-induced Fos expression in the PVN are illustrated in the photomicrographs in Figure 3.8A. Values are the group means ± SEM. As seen in Figure 3.9B, Q but not PD significantly increased Fos expression in the pPVN (One-way ANOVA followed by Dunnett's multiple comparison test, *P=0.05) compared to V. No differences in Fos expression were observed between Q, PD and V in the mPVN. Scale bars in I, III, V and II, IV, VI represent 100µm and 50µm respectively. Arrows shows Fos-positive oxytocin cells and arrowheads illustrate Fos-negative oxytocin cells 3v=third ventricle.

Figure 3.9: Effects of Quinelorane and PD168077 on Fos expression in oxytocin neurons in the subdivisions of the PVN



Effects of Vehicle (V, n=7, 2µl), Quinelorane (Q, n=8, 1µg) and PD168077 (PD, n=10, 4.5µg) on Fos expression in the subdivisions of the PVN. V and Q-induced Fos expression in the medial parvocelllular (mp) subregion of the PVN are illustrated in the photomicrographs in Figure 3.9A. Values are the group means ± SEM. As seen in Figure 3.9B, Q but not PD significantly increased Fos expression in the the mp subdivision of the PVN (One-way ANOVA followed by Dunnett's multiple comparison test, *P=0.001) compared to V. No differences in Fos expression were observed between treatment groups in any of the other PVN subdivisions. Scale bars in I, III and II, IV represent 100µm and 50µm respectively. Arrows shows Fos-positive oxytocin cells and arrowheads illustrate Fos-negative oxytocin cells 3v=third ventricle; ap=anterior parvocellular; dp=dorsal parvocellular; mp=lateral parvocellular; lp=lateral parvocellular.

did not show any significant deficits in mounting or intromitting behaviour (Figure 3.10A). Likewise, the D3A did not significantly affect mounting behaviour, however, the D3A did reduce the incidence of intromitting behaviour which was almost significant (Chi-squared test with Yates correction, P=0.09) (Figure 3.10B). The D4A did not affect the incidence of mounting, however, it did significantly decrease the incidence of intromission (Chi-squared test with Yates correction, P<0.05) (Figure 3.10C).

3.3.2.3 Effects of D2-like antagonists on mount and intromission latencies

Those rats who did not mount or intromit within the 15 min observation period were assigned a value of 900 secs and excluded from statistical analysis. Neither the D2A (1μg or 10μg), D3A (5μg) or D4A (7μg) had any significant effects on intromission latency (Mann-Whitney Rank Sum test) (Figure 3.11A-C) in those males displaying intromitting behaviour. Interestingly, the D4A but not the D2A or D3A significantly increased the latency to mount in those males displaying mounting behaviour (Mann-Whitney Rank Sum test, P<0.05) (Figure 3.11C). Latency graphs A-C in Figure 3.11 show a representative bar chart for each treatment group.

3.3.2.4 Effects D2-like antagonists on mount and intromission frequencies

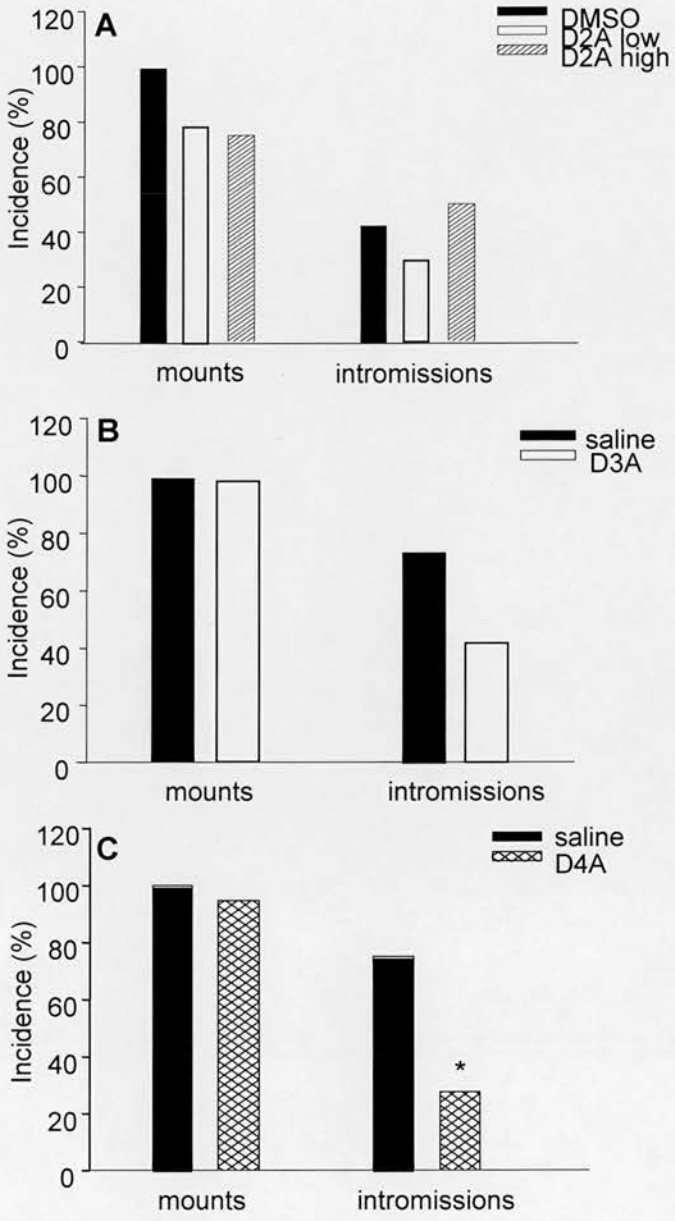
Those rats who did not mount or intromit within the 15 min observation period were assigned a value of zero and excluded from statistical analysis. Neither the D2A (1μg or 10μg), D3A (5μg) or D4A (7μg) had any significant effects on mount or intromission frequency in those males exhibiting mounting and intromitting behaviour, respectively (One-way ANOVA or Student's t-test) (Figure 3.12).

3.3.2.5. Effects of D2-like antagonists on Fos expression

MPN

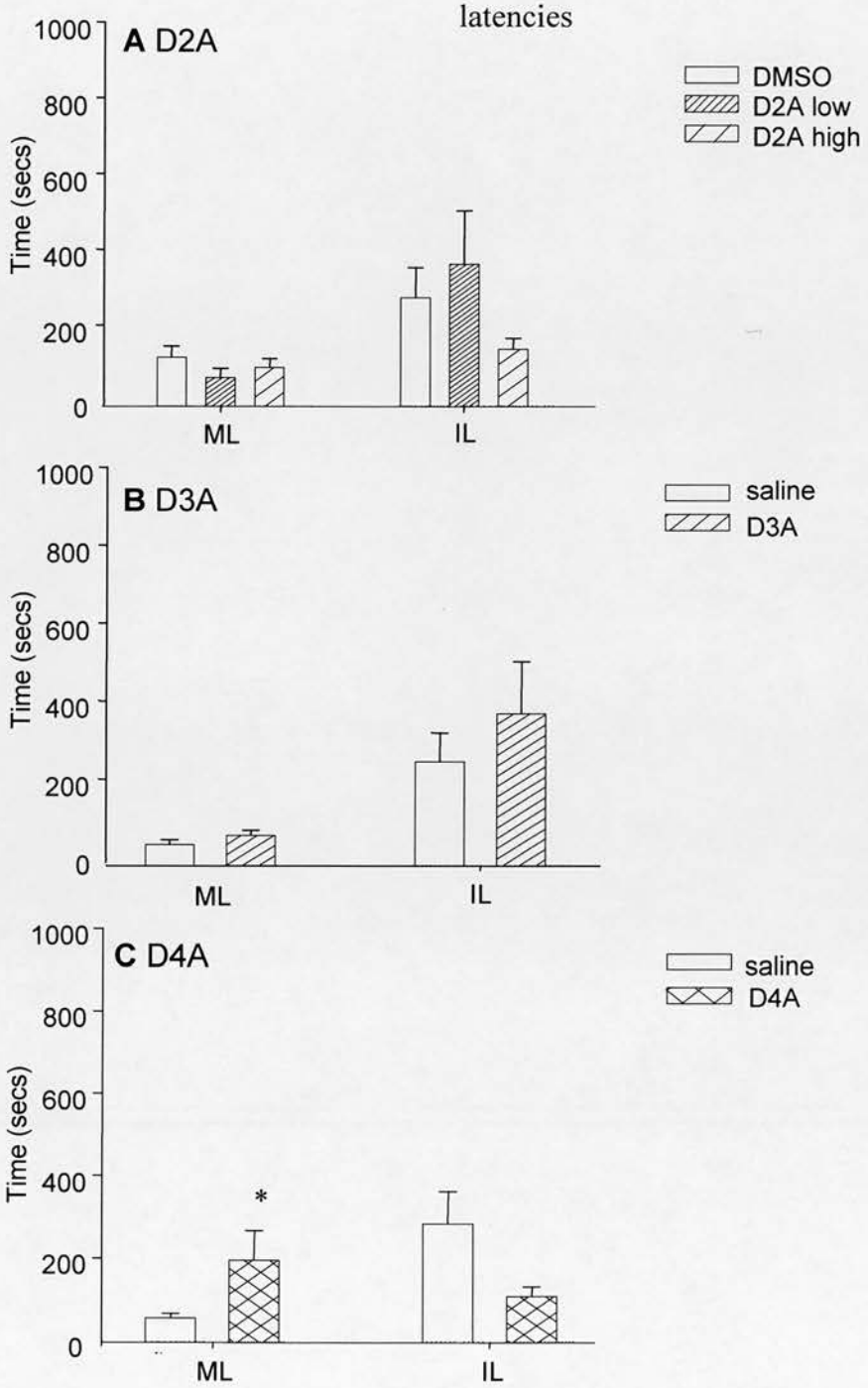
Photomicrographs shown in Figures 3.13A and 3.14A show Fos expression in the MPN after i.c.v. injection of the D2A (1μg or 10μg), D3A (5μg) or D4A (7μg) respectively of intromitting (I) males. The D3A but not the D2A (D2A low or D2A high) or D4A significantly decreased the number of Fos-positive

Figure 3.10: Effects of D2A, D3A or D4A on the incidence of mounting and intromission.



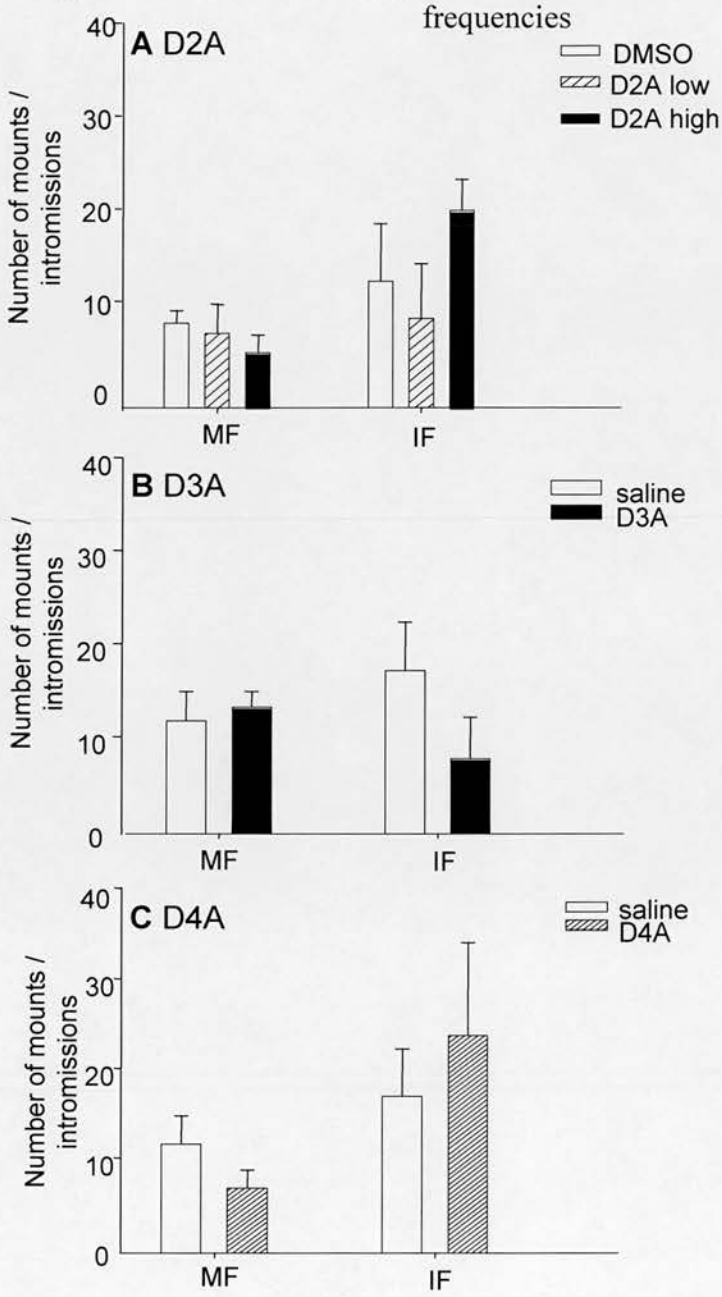
The effect of L-741,626 (D2A low, 1µg, n=10 and D2A high, 10µg, n=12) (3.10A), nafadotride (D3A, 5µg, n=12) (3.10B) , L-745,870 (D4A, 7µg, n=18) (3.10C) and the corresponding vehicle (DMSO, n=14 or saline, n=12) on the incidence (%) of mounts and intromissions in male rats placed with receptive females during 15 min observation period. Values are the group means ± SEM. Neither the D2A, D3A or D4A had any effect on the incidence of mounting. The D4A but not the D2A or D3A significantly decreased the incidence of intromission (Chi-square test with Yates correction, *P<0.05).

Figure 3.11: Effects of the D2A, D3A an D4A on mount and intromission latencies



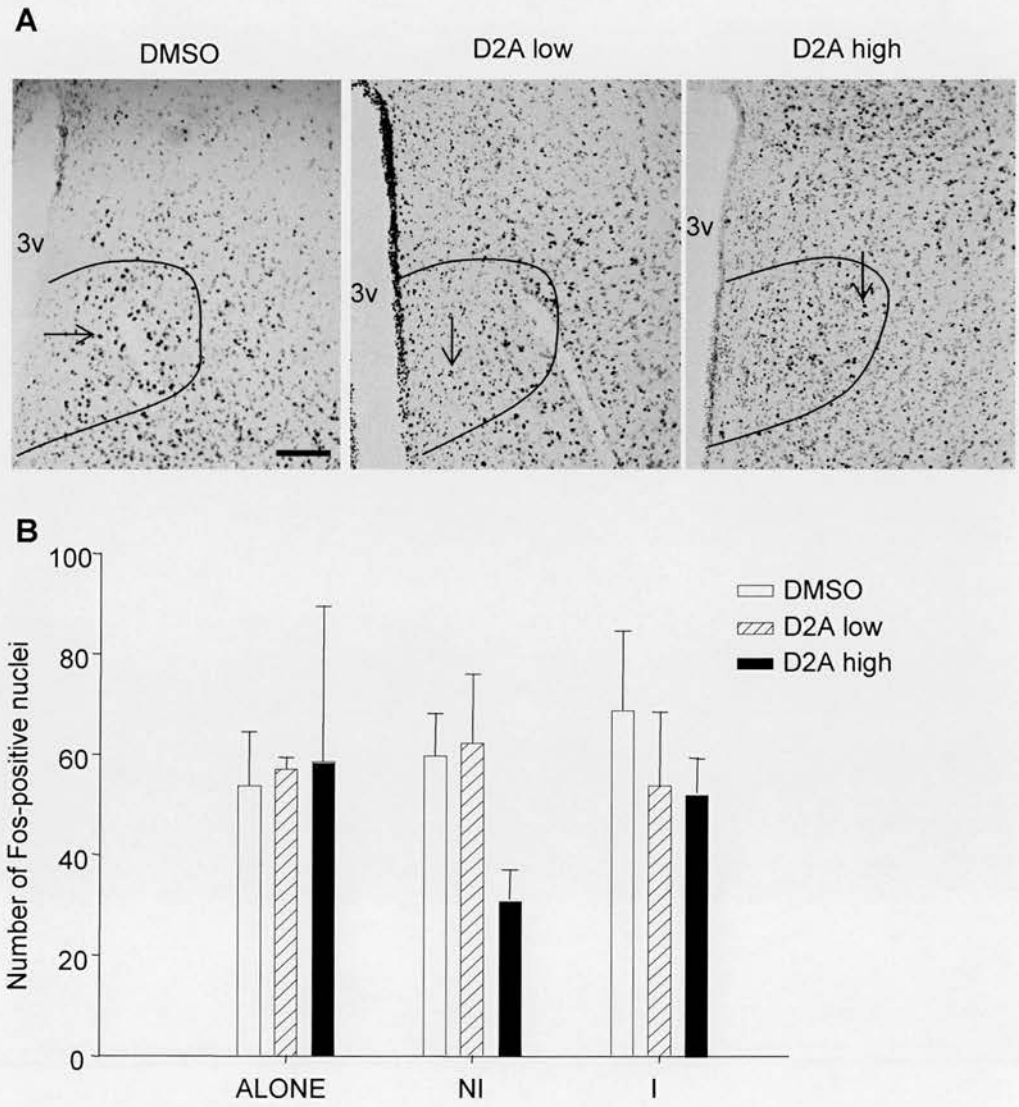
The effect of L-741,626 (D2A low, 1µg, n=10 and D2A high, 10µg, n=12) (3.11A), nafadotride (D3A, 5µg, n=12) (3.11B) , L-745,870 (D4A, 7µg, n=18) (3.11C) and the corresponding vehicle (DMSO, n=14 or saline, n=12) on mount and intromission latencies (ML and IL). Those rats that did not mount or intromit during the 15 min experimental time period were assigned a value of 900 secs and excluded from statistical analysis. Values are the group means ± SEM. The D4A but not the D2A or D3A significantly increased ML (Mann-Whitney Rank Sum test, *$P<0.05$). Neither the D2A, D3A or D4A had any effect on IL (Mann-Whitney Rank Sum test).

Figure 3.12: Effects of D2A, D3A and D4A on mount and intromission frequencies



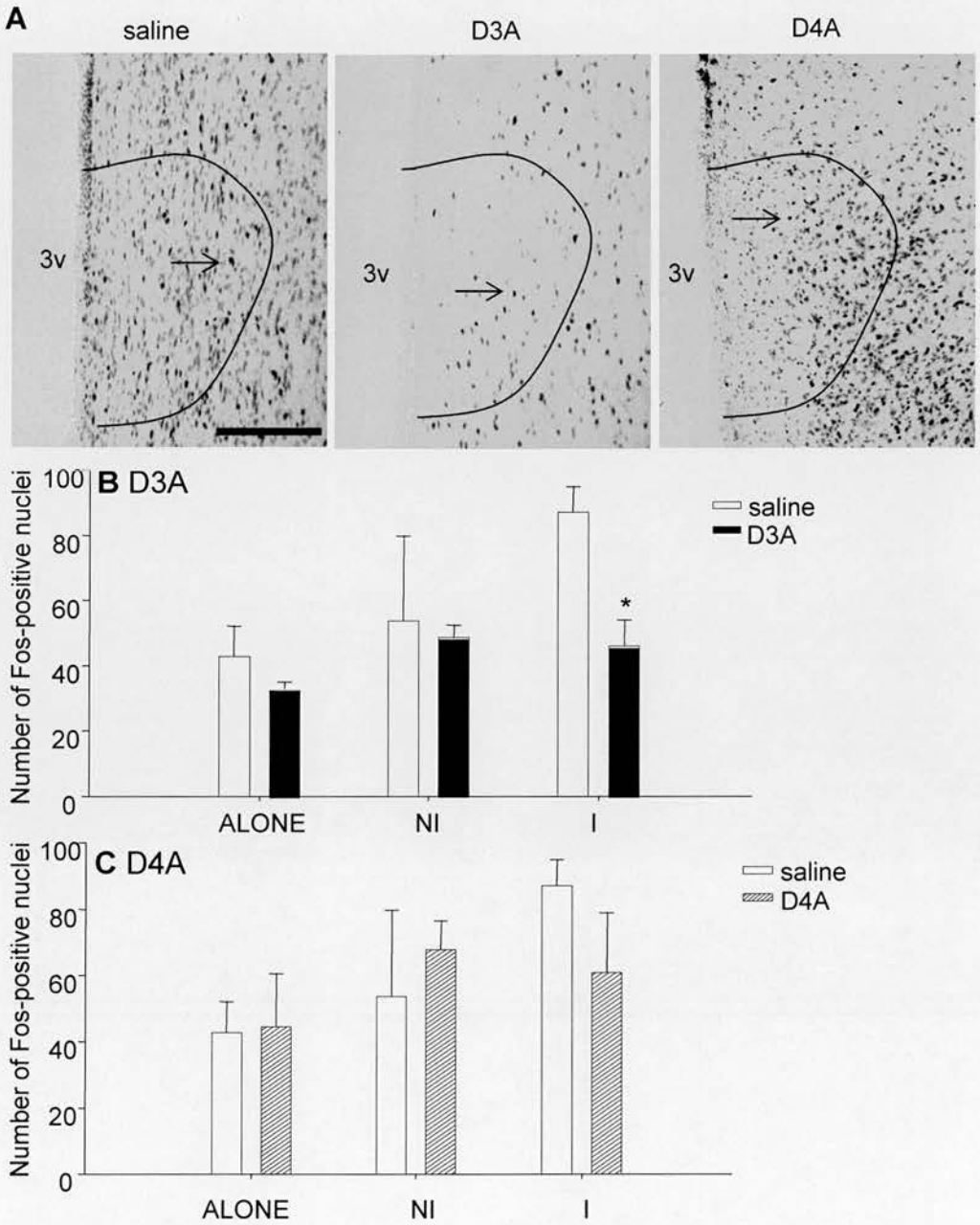
The effect of L-741,626 (D2A low, 1µg, n=10 and D2A high, 10µg, n=12) (3.12A), nafadotride (D3A, 5µg, n=12) (3.12B) , L-745,870 (D4A, 7µg, n=18) (3.12C) and the corresponding vehicle (DMSO, n=14 or saline, n=12) on mount and intromission frequencies (MF and IF, respectively). Those rats that did not mount or intromit during the 15 min experimental time period were assigned a value of zero secs and excluded from statistical analysis. Values are the group means ± SEM. Neither the D2A, D3A or D4A had any effect on MF and IF (One Way ANOVA or Student's t-test).

Figure 3.13: Effects of D2A on Fos expression in the MPN



The effect of L-741,626 (D2A low, 1µg and D2A high, 10µg) and the corresponding vehicle (DMSO, n=6) on Fos expression in the MPN of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.13A show the effect of DMSO, D2A low and D2A high on Fos expression in the MPN of I males. Values are the group means ± SEM. As seen in Figure 3.13B, neither dose of the D2A (D2A low=1µg, n=3 and D2A high=10µg, n=6) had any significant effect on Fos expression in the MPN of I males (One Way ANOVA) compared to vehicel (DMSO, n=6) . No effect between treatment groups was seen in the ALONE and NI males. Black arrows indicate Fos-positive nuclei. Scale bars represent 200µm . 3v=third ventricle.

Figure 3.14: Effects of D3A and D4A on Fos expression in the MPN



The effect of nafadotride (D3A 5µg), L-745,870 (D4A 7 µg) and the corresponding vehicle (saline) on Fos expression in the MPN of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.14A show the effect of saline, D3A and D4A on Fos expression in the MPN of I males. Values are the group means ± SEM. The D3A (n=5) but not the D4A (5) significantly decreased the Fos expression in the MPN of I males (Student's t-test, $P<0.01$) compared to vehicle (saline, n=9) (Figure 3.14B, 3.14C). Black arrows indicate Fos-positive nuclei. No effect between treatment groups was seen in the ALONE and NI males. Scale bars represent 100µm. 3v=third ventricle.

nuclei in the MPN of I male rats (Student's t-test, $P<0.01$) (Figure 3.13B and 3.14B) compared to Vehicle (DMSO or saline). No differences were detected between treatment groups in the males placed alone (ALONE) or those who did not intromit (NI).

SON

Photomicrographs shown in Figures 3.15A and 3.16A show Fos expression in the SON of intromitting (I) males, after i.c.v. injection of the D2A (1μg or 10μg), D3A (5μg) or D4A (7μg) respectively. The D3A but not the D2A (D2A low or D2A high) or D4A (n=5) significantly decreased the number of Fos-positive nuclei in the SON of I males (Student's t-test, $P<0.05$) compared to Vehicle (Figure 3.15B and 3.16B). No differences were detected between treatment groups in the males placed alone (ALONE) or those who did not intromit (NI).

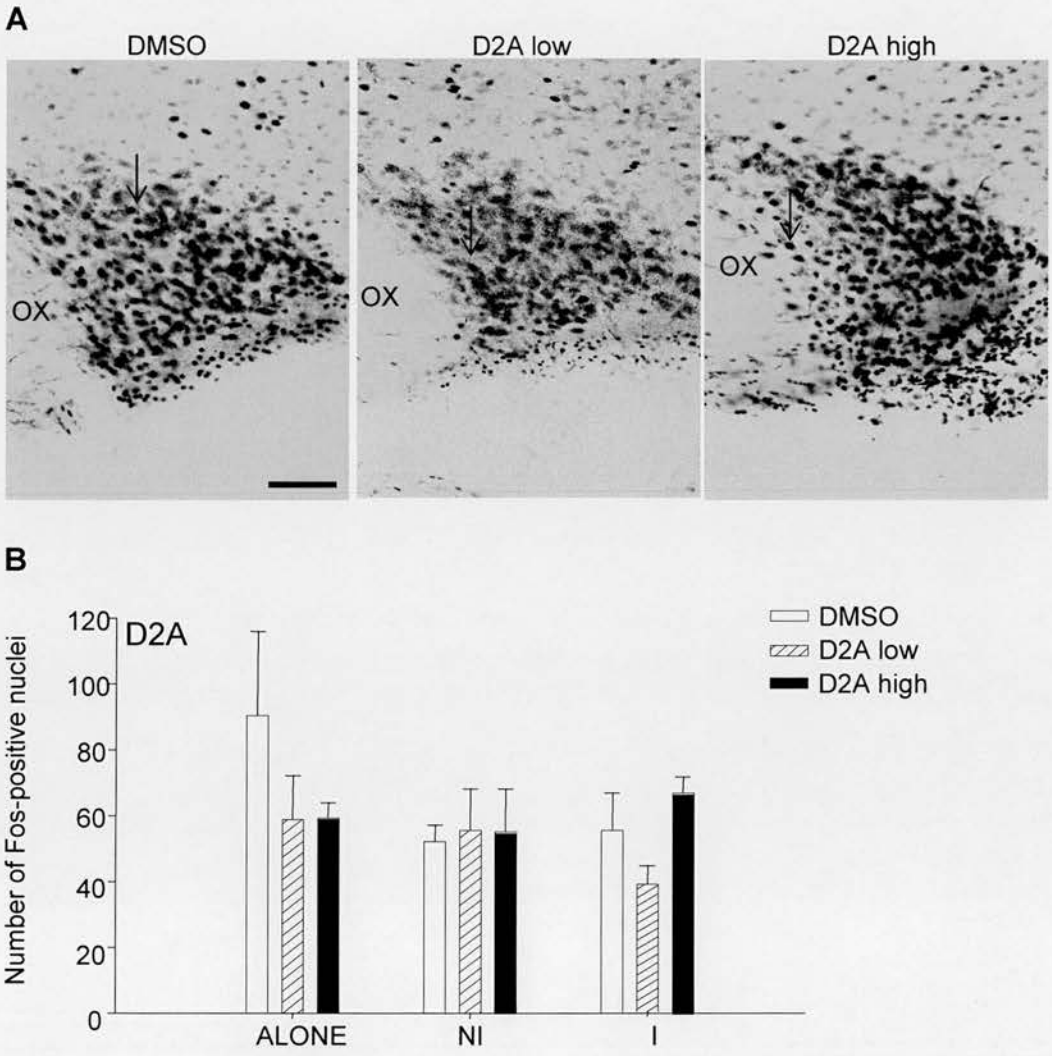
PVN

Photomicrographs shown in Figures 3.17A and 3.18A show Fos expression in the whole pPVN of intromitting (I) males, after i.c.v. injection of the D2A (1μg or 10μg), D3A (5μg) or D4A (7μg) respectively, of intromitting (I) males. The D3A but not the D2A (D2A low or D2A high) or D4A significantly decreased the number of Fos-positive nuclei in the pPVN of I male rats (Student's t-test, $P<0.05$) compared to vehicle (DMSO, n=6 or saline, n=9) (Figure 3.17B and3.18B). No differences were detected between treatment groups in the males placed alone (ALONE) or those who did not intromit (NI).

PVN subdivisions

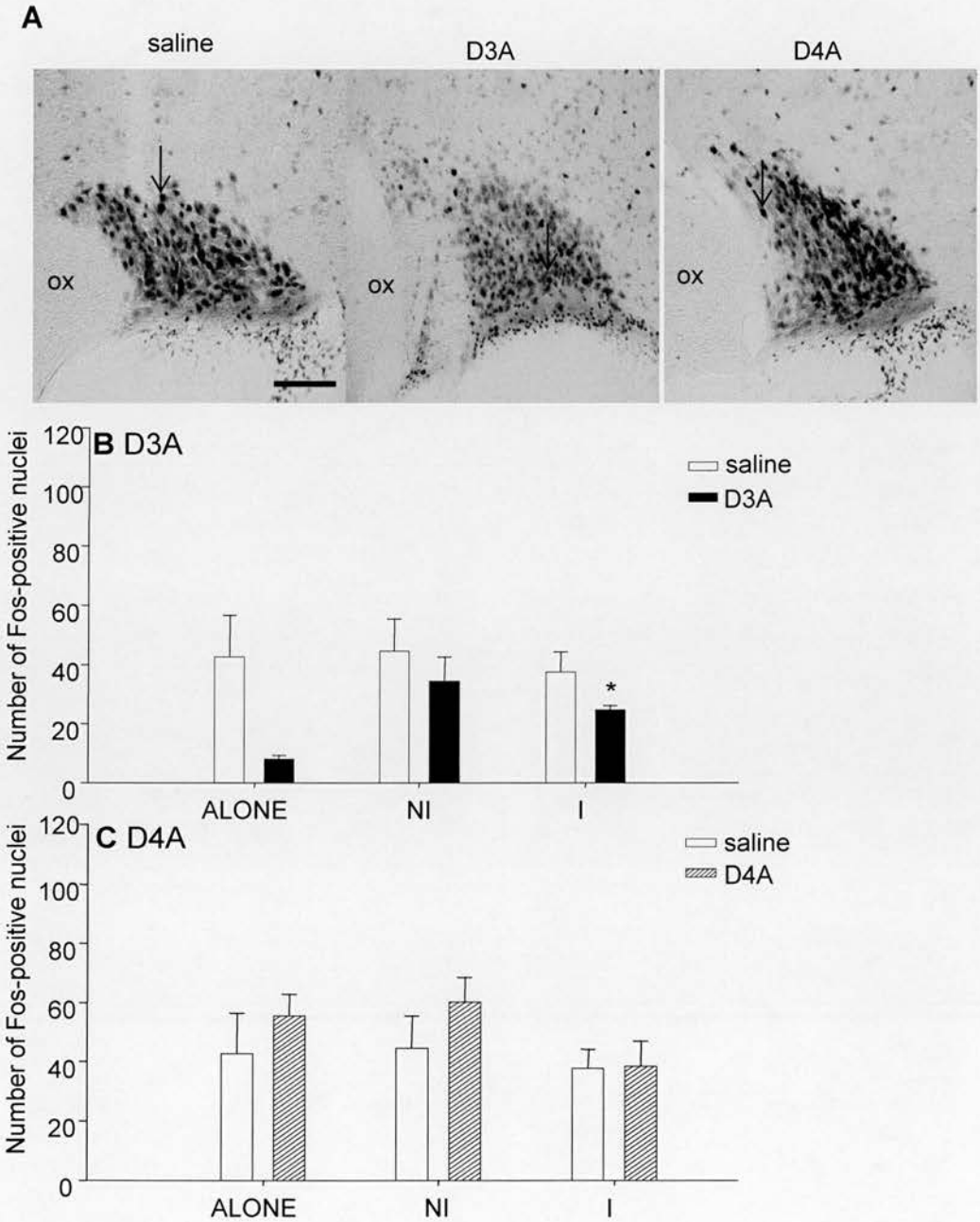
Figure 3.19 displays Fos expression in the individual subregions of the pPVN after i.c.v. injection of the D2A (1μg or 10μg) in intromitting rats only. The high dose (10μg, n=6) but not the low dose (1μg, n=3) of the D2A significantly increased the number of Fos-positive nuclei in the anterior parvocellular subdivision of the PVN of I males (One-way ANOVA followed by Dunnett's multiple comparison test; $*P<0.05$) compared to vehicle (DMSO, n=6). No other treatment effects were observed in any of the

Figure 3.15: Effects of D2A on Fos expression in the SON



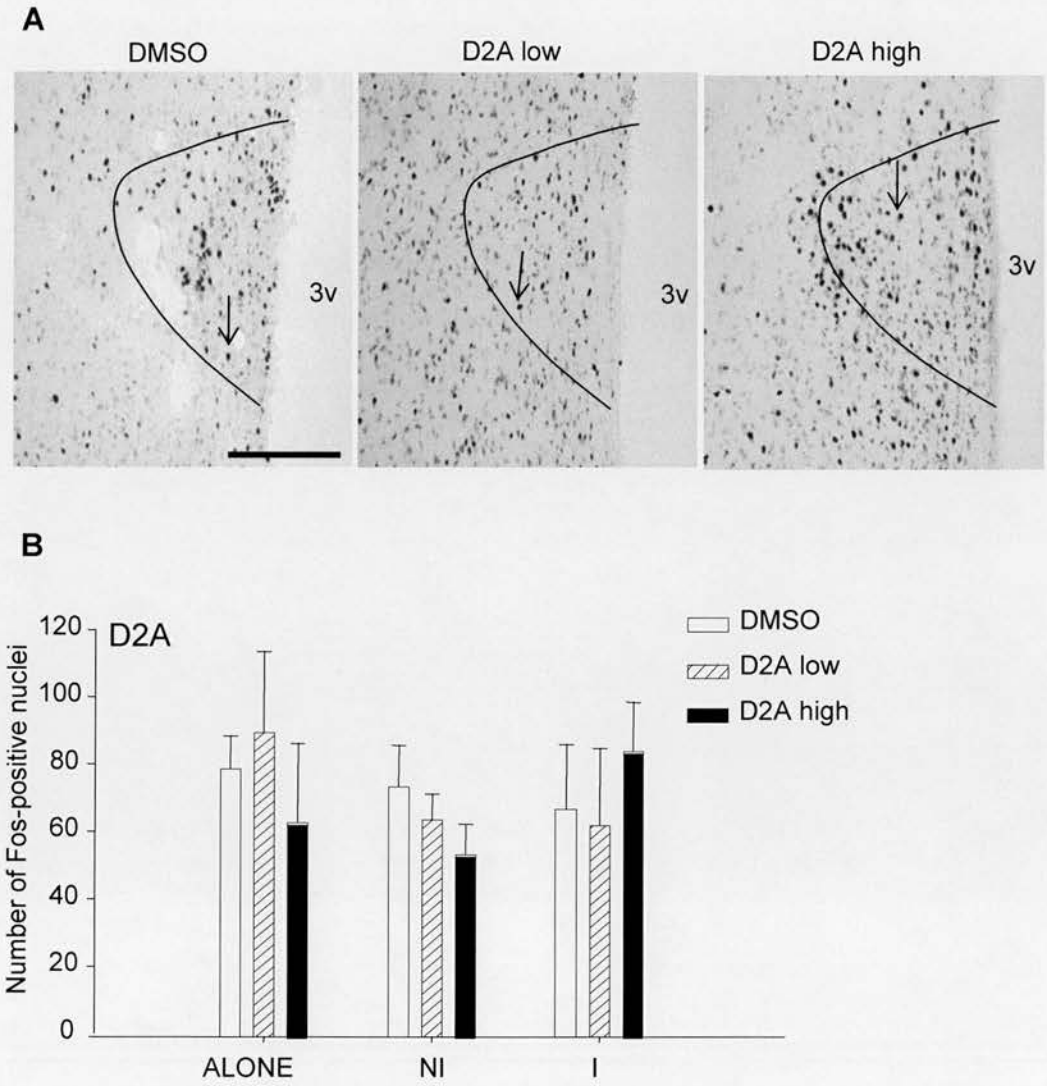
The effect of L-741,626 (D2A low, 1μg and D2A high, 10μg) and the corresponding vehicle (DMSO) on Fos expression in the SON of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.15A show the effect of DMSO, D2A low and D2A high on Fos expression in the SON of I males. Values are the group means ± SEM. Neither dose of the D2A (D2A low=1μg, n=3 and D2A high=10μg, n=6) had any significant effect on Fos expression in the SON of I males (One Way ANOVA) compared to vehicel(DMSO, n=6) (Figure 3.15B). No effect between treatment groups was seen in the ALONE and NI males. Black arrows indicate Fos-positive nuclei. Scale bars represent 100μm . OX=optic chiasm.

Figure 3.16: Effects of D3A and D4A on Fos expression in the SON



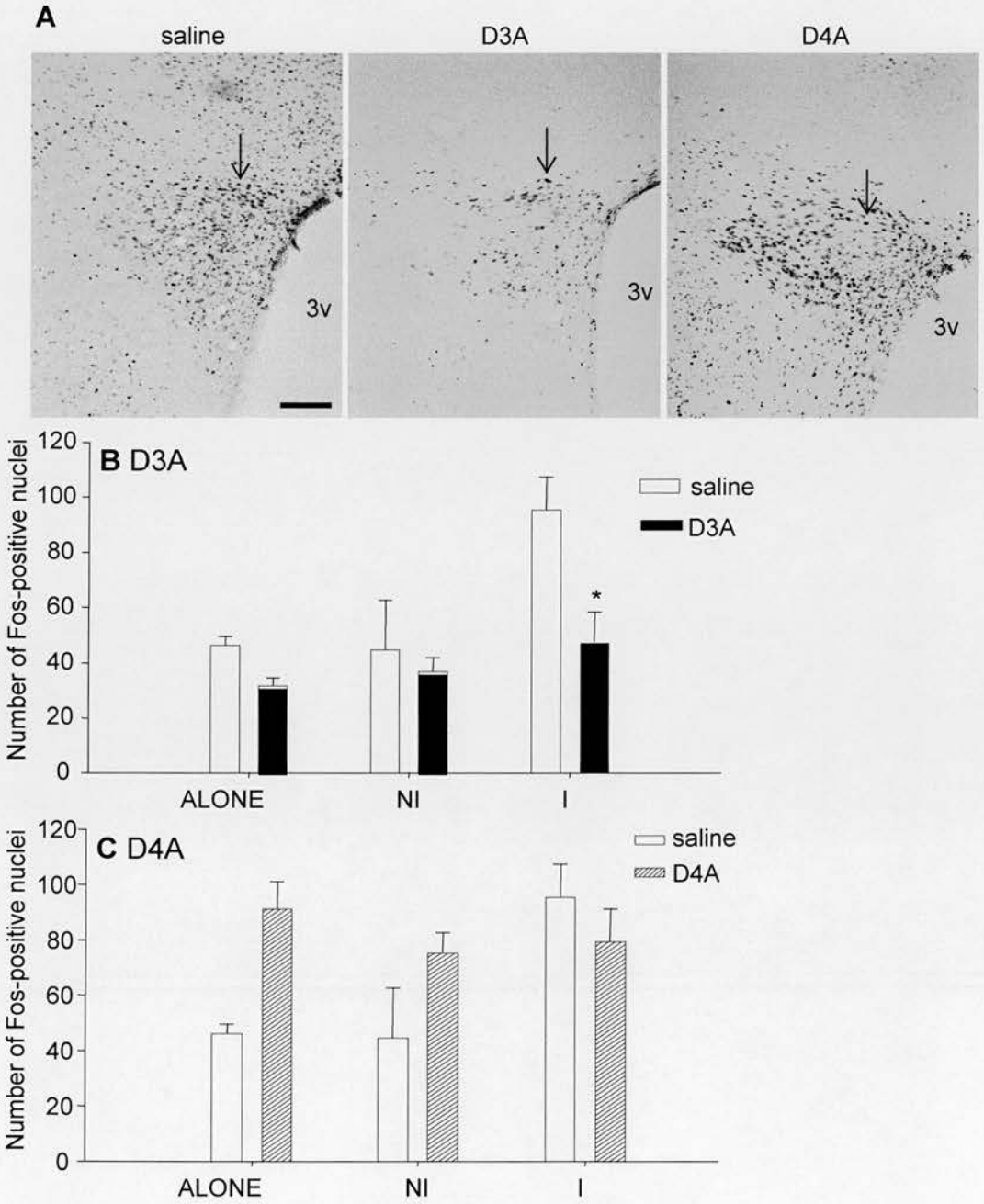
The effect of nafadotride (D3A 5µg), L-745,870 (D4A 7 µg) and the corresponding vehicle (saline) on Fos expression in the SON of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.16A show the effect of saline, D3A and D4A on Fos expression in the SON of I males. Values are the group means ± SEM. The D3A (n=5) but not the D4A (n=5) significantly decreased the Fos expression in the SON of I males (Student's t-test, $P<0.05$) compared to vehicle (saline, n=9) (Figure 3.16B, 3.16C). Black arrows indicate Fos-positive nuclei. No significant effect between treatment groups was seen in the ALONE and NI males. Scale bars represent 100µm. OX=optic chiasm.

Figure 3.17: Effects of D2A on Fos expression in the whole pPVN



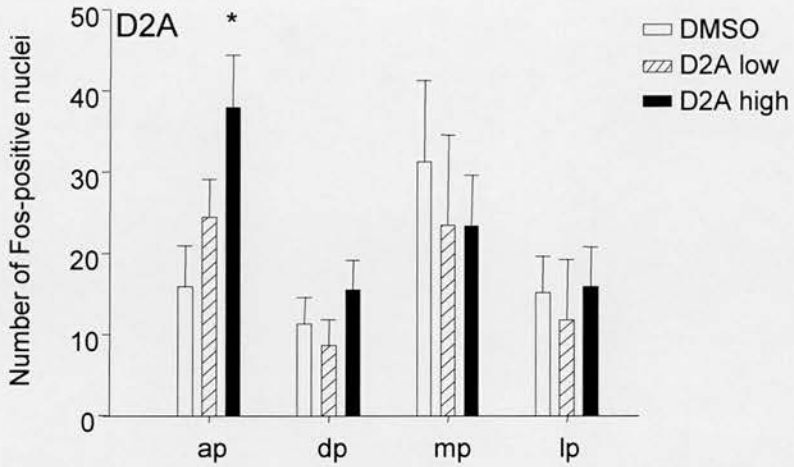
The effect of L-741,626 (D2A low, 1μg and D2A high, 10μg) and the corresponding vehicle (DMSO) on Fos expression in the PVN of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.17A show the effect of DMSO, D2A low and D2A high on Fos expression in the anterior PVN of I males. Values are the group means ± SEM. Neither dose of the D2A (D2A low=1μg, n=3 and D2A high=10μg, n=6) had any significant effect on Fos expression in the PVN of I males (One Way ANOVA) compared to vehicle (DMSO, n=6) (Figure 13.7B). No effect between treatment groups was seen in the ALONE and NI males. Black arrows indicate Fos-positive nuclei. Scale bars represent 100μm . 3v=third ventricle.

Figure 3.18: Effects of D3A and D4A on Fos expression in the PVN



The effect of nafadotride (D3A 5µg), L-745,870 (D4A 7 µg) and the corresponding vehicle (saline) on Fos expression in the PVN of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.18A show the effect of saline, D3A and D4A on Fos expression in the PVN of I males. Values are the group means ± SEM. The D3A (n=5) but not the D4A (n=5) significantly decreased Fos expression in the pPVN of I males (Student's t-test, $P<0.05$) compared to vehicle (saline, n=9) (Figure 3.18B, 3.18C). No effect between treatment groups was seen in the ALONE and NI males. Black arrows indicate Fos-positive nuclei. Scale bars represent 200µm. 3v=third ventricle.

Figure 3.19: Effects of D2A on Fos expression in the subdivisions of the PVN



The effect of L-741,626 (D2A low, 1µg and D2A high, 10µg) and the corresponding vehicle (DMSO) on Fos expression in the subdivisions of the PVN of intromitting (I) males. Values are the group means ± SEM. The high dose (10µg, n=6) but not the low dose (1µg, n=3) of the D2A significantly increased the number of Fos-positive nuclei in the ap subdivision of the PVN of I males (One-way ANOVA followed by Dunnett's multiple comparison test; *P<0.05) compared to vehicle (DMSO, n=6). No other treatment effects were observed in any of the other subregions. ap=anterior parvocellular; dp=dorsal parvocellular; mp=medial parvocellular; lp=lateral parvocellular.

other subregions. No differences were observed in ALONE and NI rats after D2A, D3A or D4A (data not shown).

Photomicrographs in Figure 3.20A illustrate Fos expression in the dorsal and medial parvocellular subdivisions of the pPVN after i.c.v. injection of the D3A (5μg) or vehicle (saline) in intromitting (I) males. The D3A but not the D4A significantly inhibited the number of Fos-positive nuclei expressed in the dorsal and medial parvocellular subregions of the pPVN in I males (Student's t-test; *P<0.05) compared to vehicle (saline, n=9) (Figure 3.20B). Additionally, the antagonistic effect of the D3A in the lateral parvocellular subregion was almost significant (Student's t-test, P=0.07). No treatment effect was observed in the anterior parvocellular subregion. The D4A had no effect on Fos expression in any of the subregions. Additionally, no differences in Fos expression in ALONE and NI rats after D2A, D3A or D4A were observed (data not shown).

mPVN

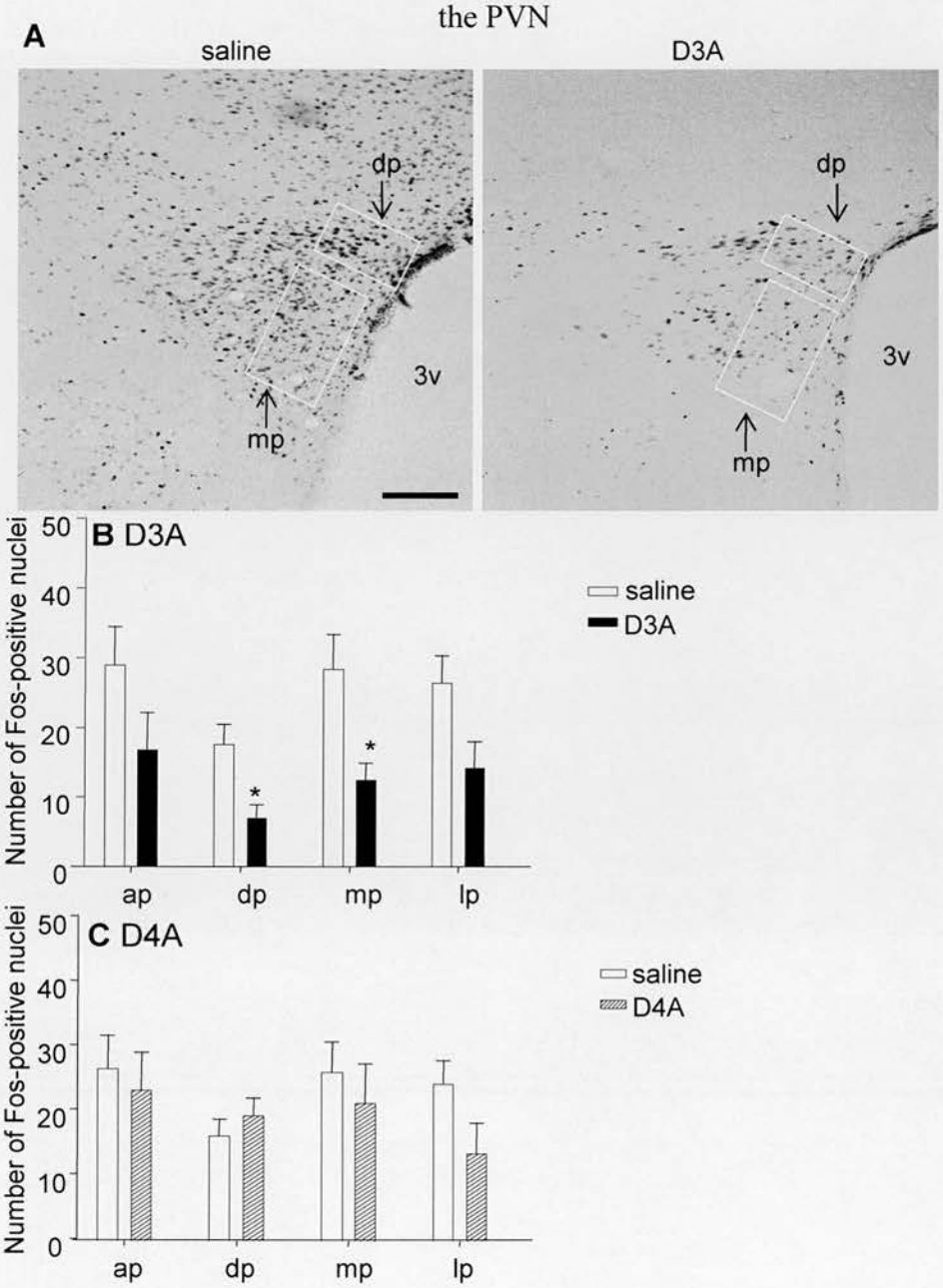
Graphs displayed in Figure 3.21 show Fos expression in the mPVN after i.c.v. injection of the D2A (1μg or 10μg), D3A (5μg) or D4A (7μg) respectively, in intromitting (I) males. Neither the D2A (D2A low, n=3 or D2A high, n=6), D3A (n=5) or D4A (n=5) had any significant effect on Fos expression in the mPVN of I males (One Way ANOVA or Student's t-test) compared to corresponding vehicle (DMSO, n=6 or saline, n=9). No differences were detected between treatment groups in the males placed alone (ALONE) or those who did not intromit (NI).

3.3.2.6 Effects of D2-like antagonists on Fos expression in oxytocin neurons

MPN

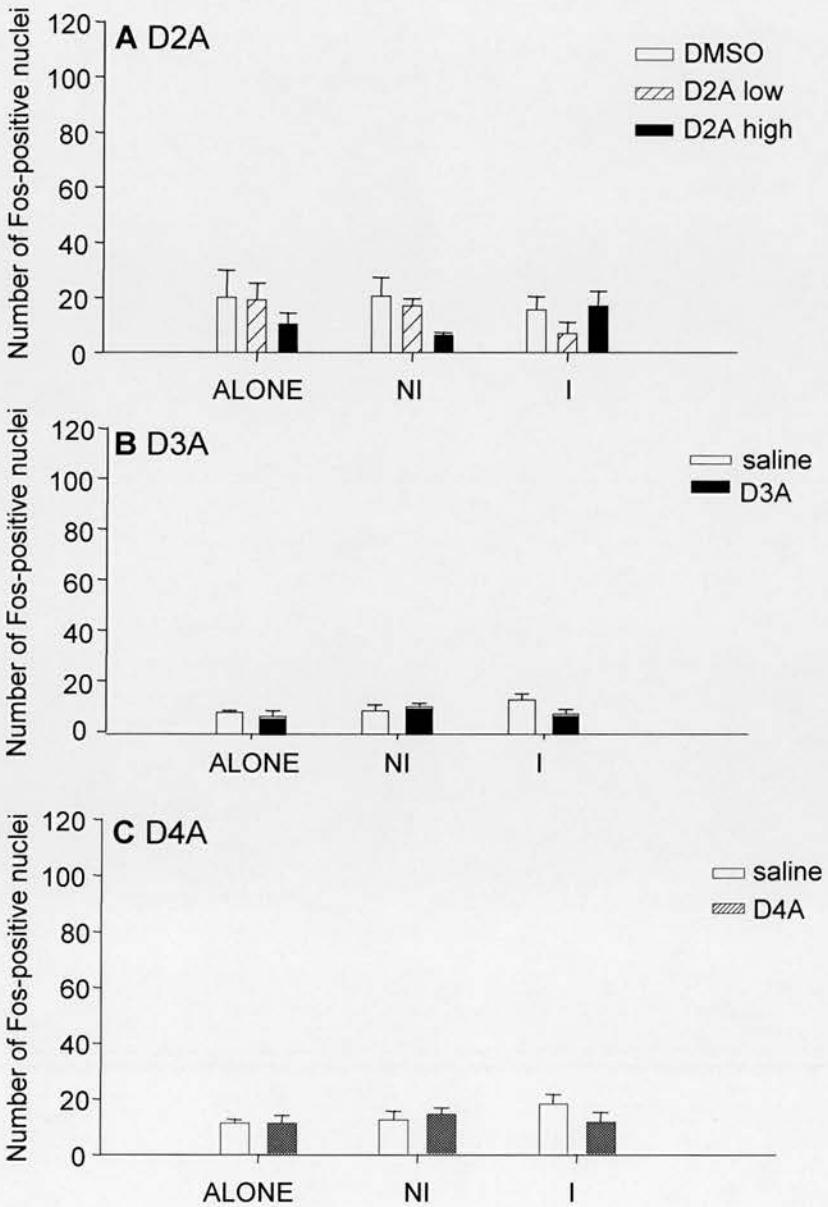
Photomicrographs shown in Figures 3.22 (A,B), 3.23 (A,B) and 3.24 (A,B) show Fos expression in oxytocin neurons in the MPN after i.c.v. injection of the D2A (1μg or 10μg), D3A (5μg) or D4A (7μg), respectively, of intromitting (I) males. Neither D2A (D2A low or D2A high) (Figure 3.22C), D3A (Figure

Figure 3.20: Effects of D3A and D4A on Fos expression in the subdivisions of the PVN



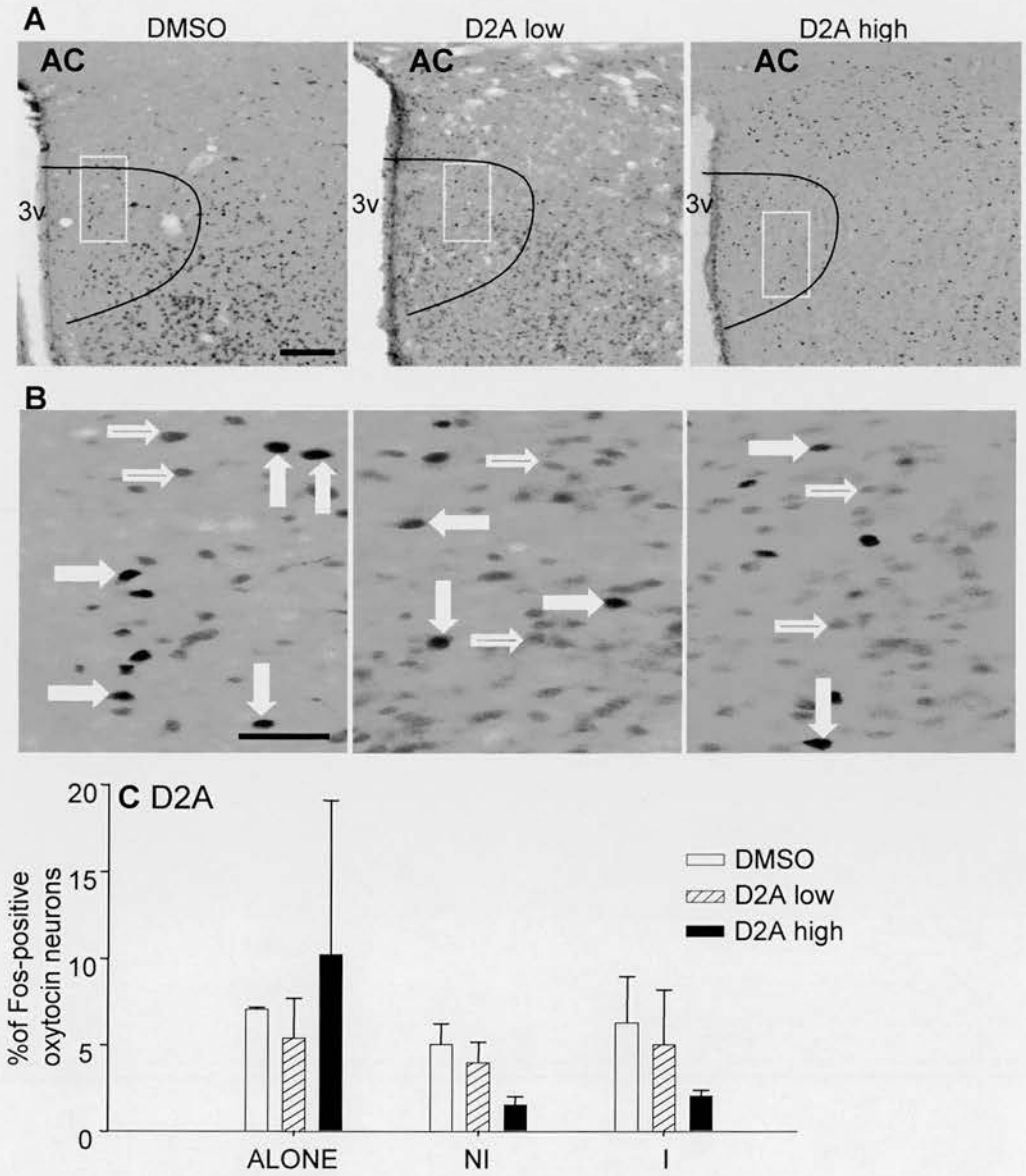
The effect of nafadotride (D3A 5µg), L-745,870 (D4A 7 µg) and the corresponding vehicle (saline) on Fos expression in the subdivisions of the PVN of intromitting (I) males. Photomicrographs in Figure 3.20A show the effect of saline and D3A on Fos expression in the dorsal and medial pPVN. Values are the group means ± SEM. The D3A (n=5) but not the D4A (n=5) significantly decreased Fos expression in the dorsal and medial pPVN of I males (Student's t-test, $P<0.05$) compared to vehicle (saline, n=9) (Figure 3.20B, 3.20C). No effect between treatment groups was seen in the other subdivisions. Scale bars represent 200µm. 3v=third ventricle; ap=anterior parvocellular; dp=dorsal parvocellular; mp=medial parvocellular; lp=lateral parvocellular.

Figure 3.21: Effects of the D2A, D3A and D4A on Fos expression in the mPVN



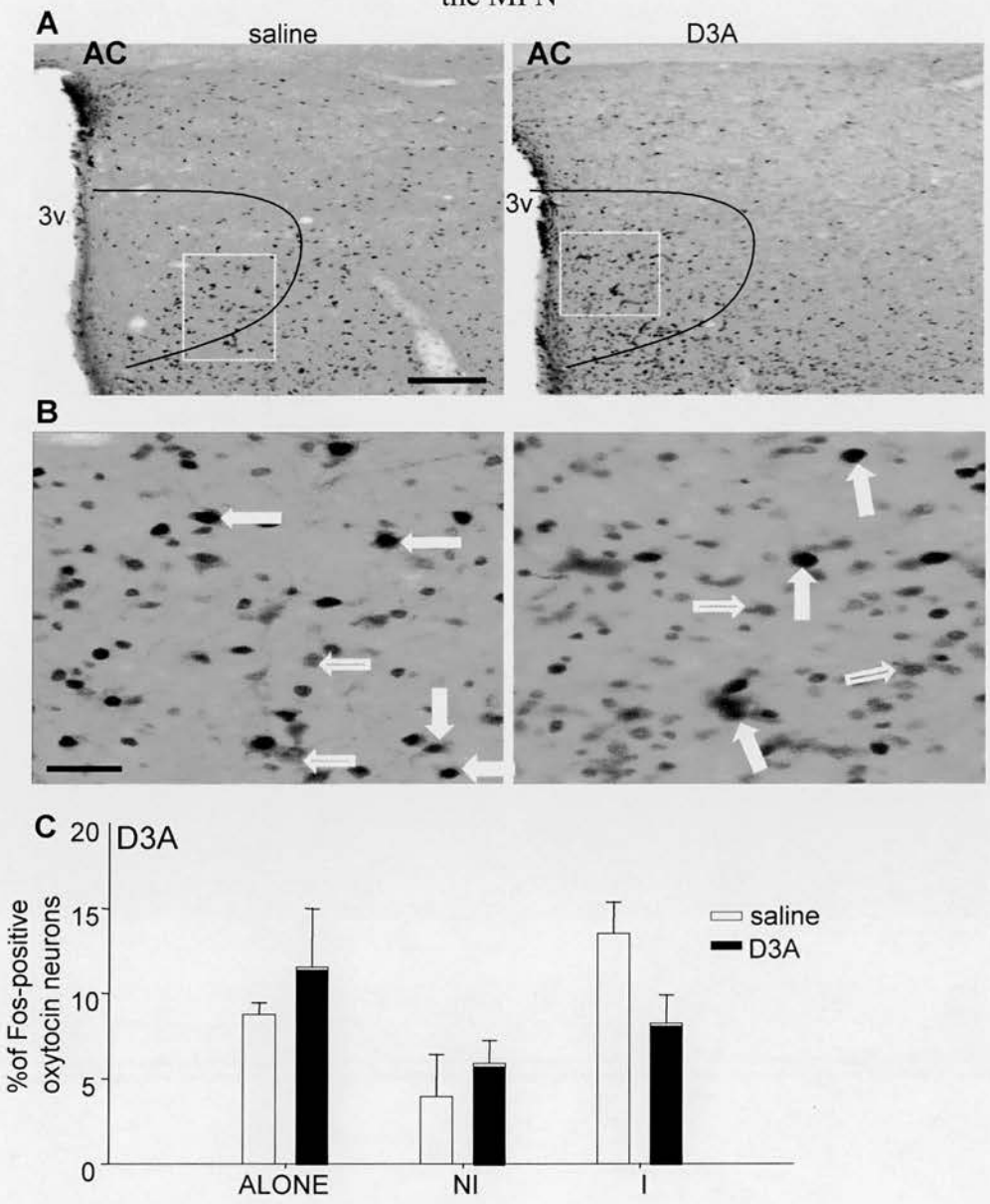
The effect of L-741,626 (D2A low, 1µg and D2A high, 10µg) (3.20A), nafadotride (D3A 5µg, n=5) (3.20B), L-745,870 (D4A 7 µg, n=5) (3.20C) and the corresponding vehicle (DMSO or saline) on Fos expression in the mPVN of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Values are the group means ± SEM. Neither the D2A (D2A low, n=3 or D2A high,n=6) D3A (n=5)or D4A (n=5) had any significant effect on Fos expression in the mPVN of I males (One Way ANOVA or Student's t-test) compared to corresponding vehicle (DMSO, n=6 or saline, n=9). No effect between treatment groups was seen in the ALONE and NI males.

Figure 3.22: Effect of the D2A on Fos expression in oxytocin neurons in the MPN



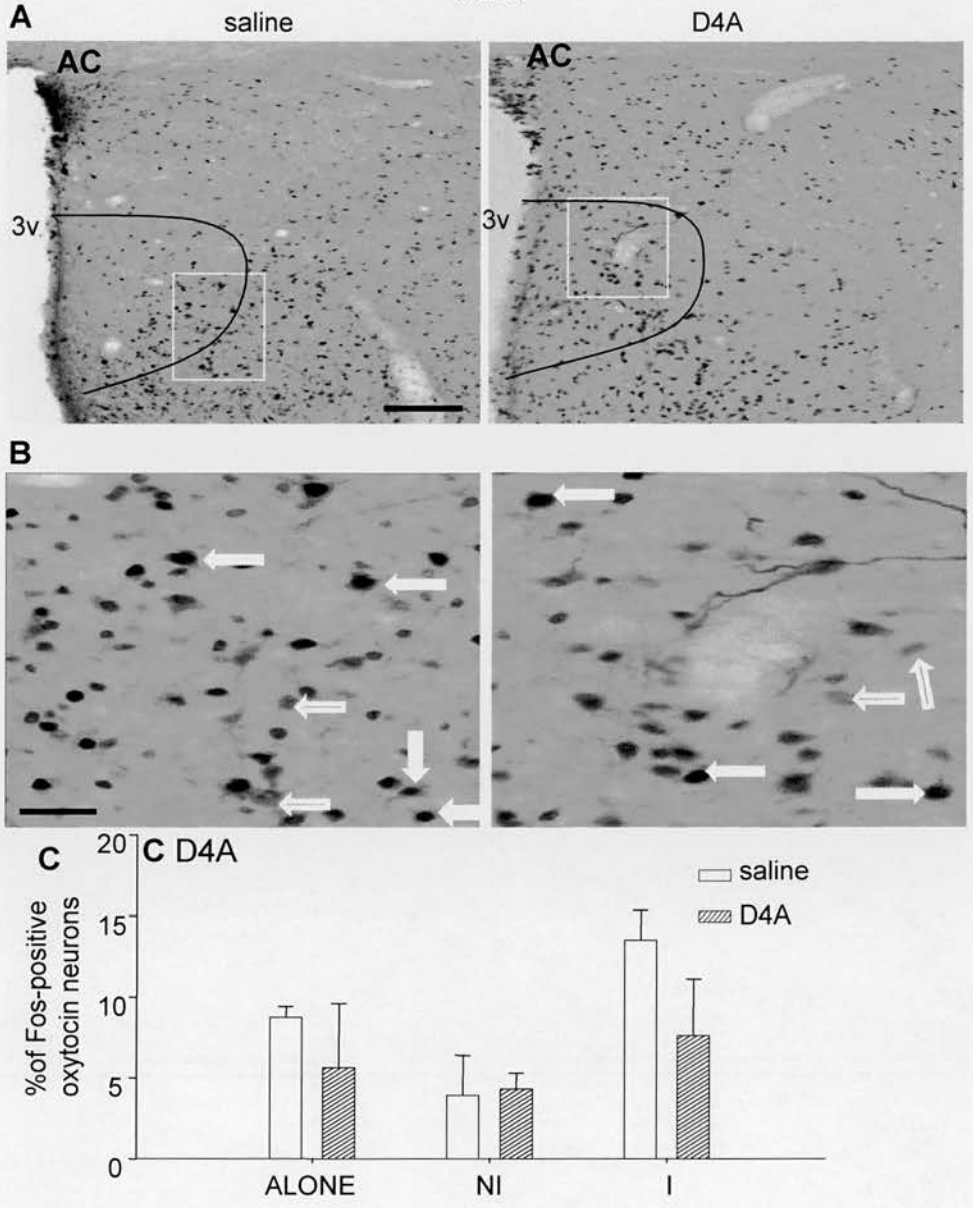
The effect of L-741,626 (D2A low, 1µg and D2A high, 10µg) and the corresponding vehicle (DMSO) on Fos expression in oxytocin cells in the MPN of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.22A and 3.22B (magnification of highlighted areas in 3.22A) show the effect of DMSO, D2A low and D2A high on Fos expression in oxytocin cells in the MPN of I males. Values are the group means ± SEM. Neither dose of the D2A (D2A low=1µg, n=3 and D2A high=10µg, n=6) had any significant effect on Fos expression in oxytocin cells in the MPN of I males (One Way ANOVA) compared to vehicle (DMSO, n=6) (Figure 3.22C). Filled arrows show a Fos-positive oxytocin cell and empty arrows show a Fos-negative oxytocin cell. No effect between treatment groups was seen in the ALONE and NI males. Scale bars represent 200µm and 50µm in 3.22A and B respectively. AC=anterior commissure; 3v=third ventricle.

Figure 3.23: Effects of the D3A on Fos expression in oxytocin neurons in the MPN



The effect of nafadotride (D3A , 5µg) and the corresponding vehicle (saline) on Fos expression in oxytocin cells in the MPN of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.23A and 3.23B (magnification of highlighted areas in 3.23A) show the effect of saline and D3A on Fos expression in oxytocin cells in the MPN of I males. Values are the group means ± SEM. The D3A (n=5) did not significantly decrease the percentage of Fos-positive oxytocin cells in the MPN of I males (Student's t-test) compared to vehicle (saline, n=5) (Figure 3.23C). Filled arrows show a Fos-positive oxytocin cell and empty arrows show a Fos-negative oxytocin cell. No effect between treatment groups was seen in the ALONE and NI males. Scale bars represent 200µm and 50µm in 3.23A and B respectively. AC=anterior commissure; 3v=third ventricle.

Figure 3.24: Effect of the D4A on Fos expression in oxytocin neurons in the MPN



The effect of L-745, 870 (D4A , 7μg) and the corresponding vehicle (saline) on Fos expression in oxytocin cells in the MPN of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.24A and 3.24B (magnification of highlighted areas in 3.23A) show the effect of saline and D4A on Fos expression in oxytocin cells in the MPN of I males. Values are the group means ± SEM. The D4A (n=5) did not significantly decrease the percentage of Fos-positive oxytocin cells in the MPN of I males (Student's t-test) compared to vehicle (saline, n=9) (Figure 3.23C). Filled arrows show a Fos-positive oxytocin cell and empty arrows show a Fos-negative oxytocin cell. No effect between treatment groups was seen in the ALONE and NI males. Scale bars represent 200μm and 50μm in 3.24A and B respectively. AC=anterior commissure; 3v=third ventricle.

3.23C) or D4A (Figure 3.24C) significantly decreased the percentage of Fos-positive oxyocin cells expressed in the MPN of intromitting (I) males (One Way ANOVA or Student's t-test) compared to vehicle (DMSO, n=6 or saline, n=9). No differences were detected between treatment groups in the males placed alone (ALONE) or those who did not intromit (NI).

SON

Photomicrographs shown in Figures 3.25A and 3.26A show Fos expression in oxytocin neurons in the SON after i.c.v. injection of the D2A (1µg or 10µg), D3A (5µg) or D4A (7µg) respectively of intromitting (I) males. The D4A (Figure 3.26C) but not the D2A (D2A low or D2A high) (Figure 3.25B) or D3A (Figure 3.26B) significantly decreased the percentage of Fos-positive oxytocin cells expressed in the SON of I males (Student's t-test, $P<0.05$) compared to vehicle (DMSO, n=6 or saline, n=9). No differences were detected between treatment groups in the males placed alone (ALONE) or those who did not intromit (NI).

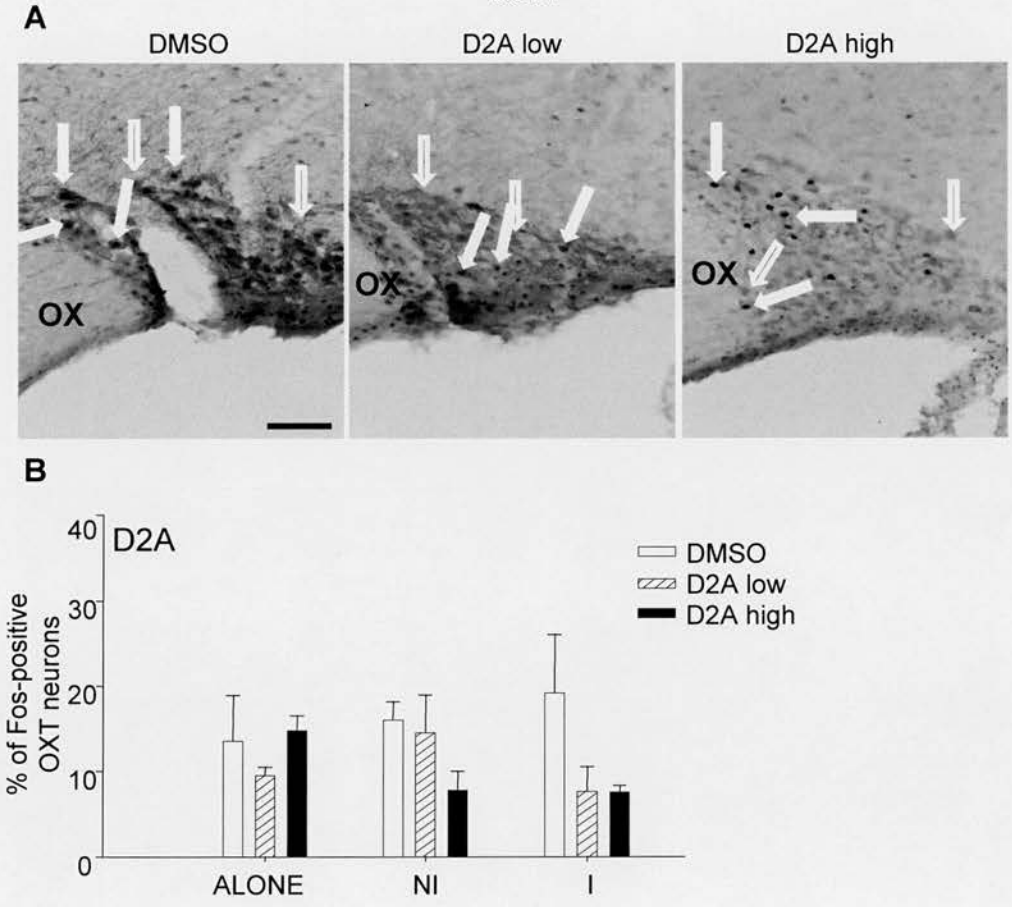
PVN

Photomicrographs shown in Figures 3.27, 3.28 and 3.29 (A-B) show Fos expression in oxytocin neurons in the PVN after i.c.v. injection of the D2A (1µg or 10µg), D3A (5µg) or D4A (7µg) respectively of intromitting (I) males. Neither the D2A (D2A low or D2A high) (Figure 3.27C), D3A (Figure 3.28C) or D4A (Figure 3.29C) had any significant effect on the percentage of Fos-positive oxytocin cells expressed in the pPVN of I males (One Way ANOVA or Student's t-test) compared to vehicle (DMSO or saline). No differences were detected between treatment groups in the males placed alone (ALONE) or those who did not intromit (NI).

PVN subdivisions

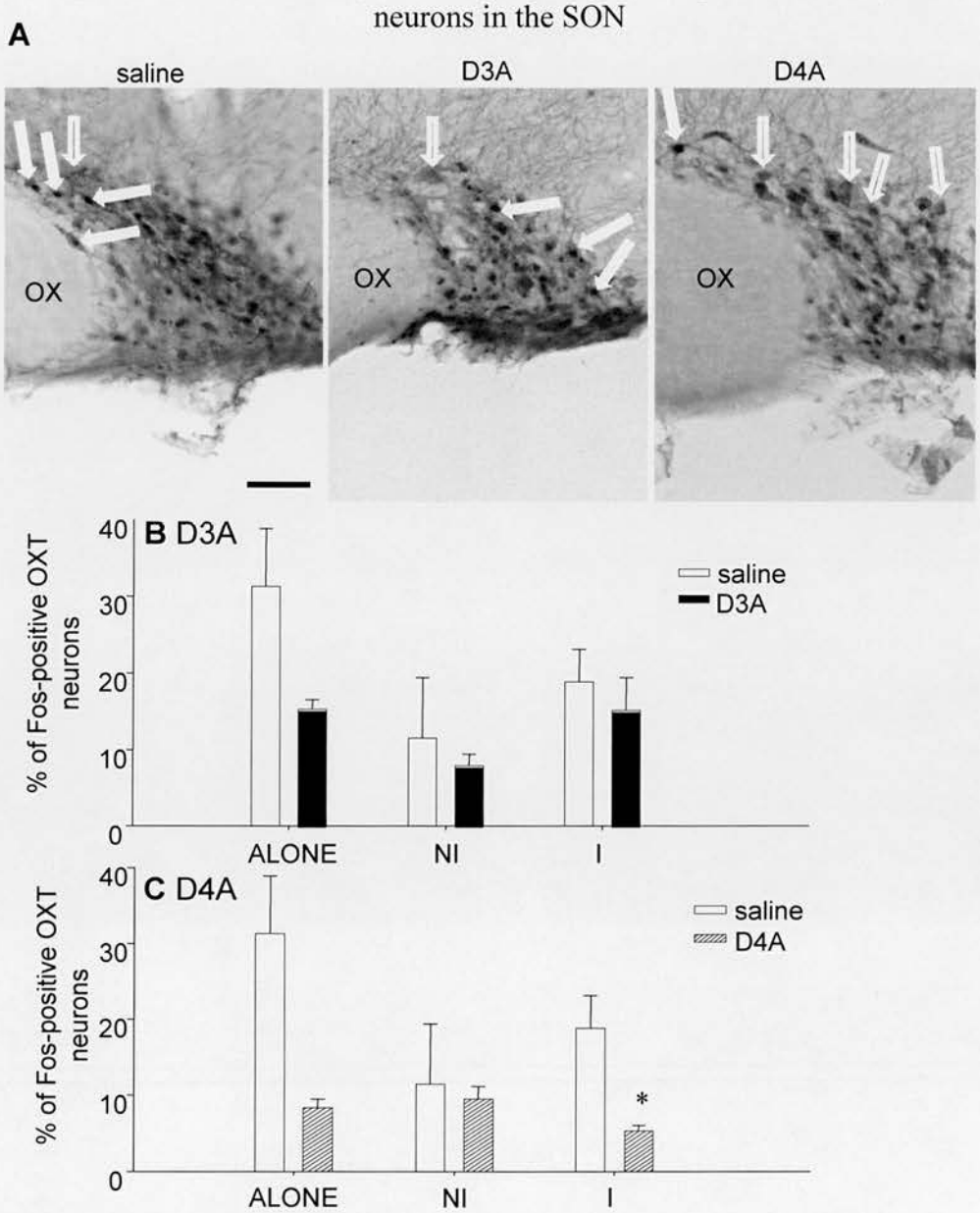
Figure 3.30 displays the percentage of Fos-positive oxytocin cells in the individual subregions of the pPVN after i.c.v. injection D2A (1µg or 10µg) (Figure 3.30A), D3A (5µg) (Figure 3.30B) or D4A (7µg) (Figure 3.30C) respectively of intromitting (I) males. Neither the D2A (D2A low or D2A high), D3A or

Figure 3.25: Effect of the D2A on Fos expression in oxytocin neurons in the SON



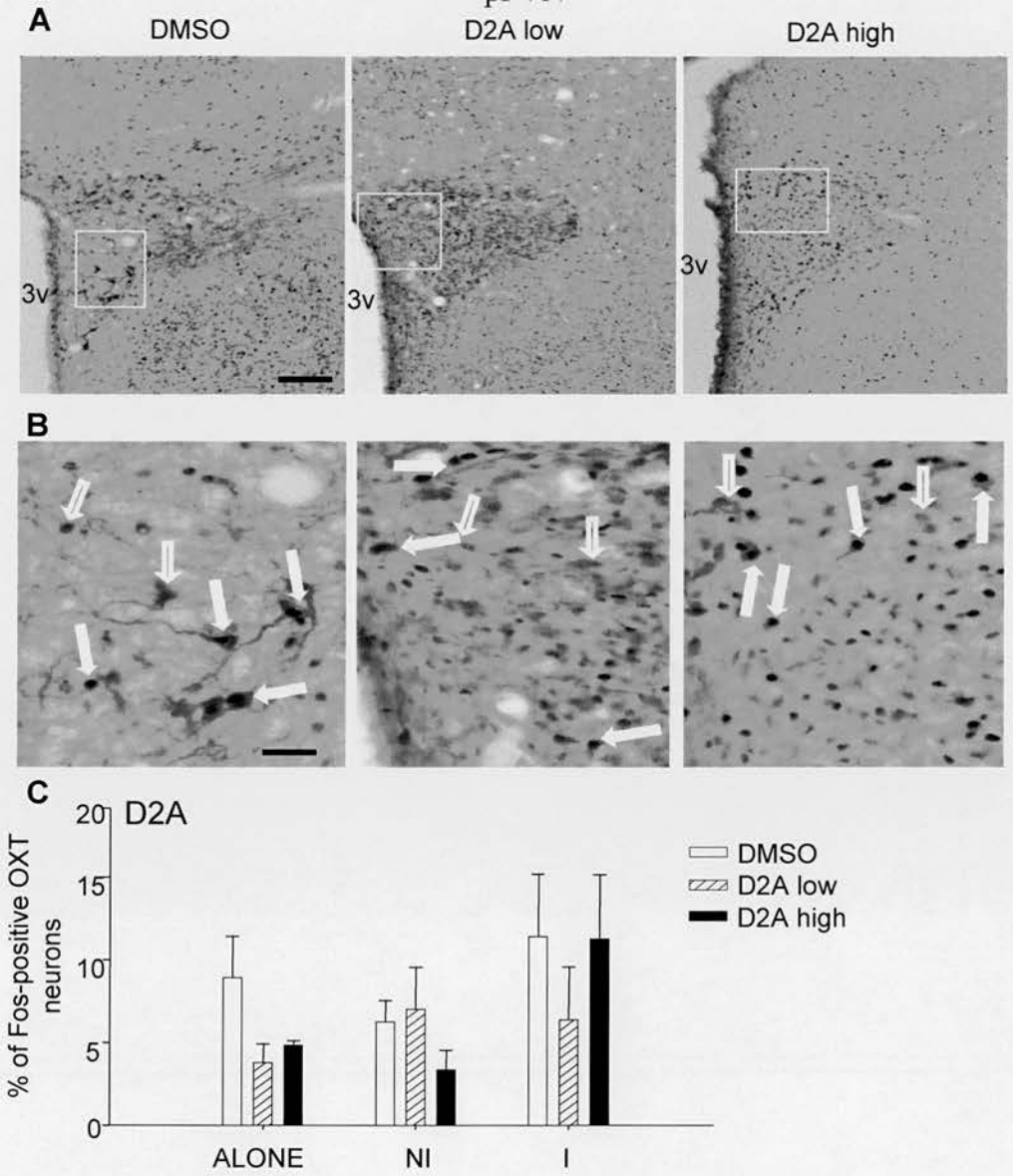
The effect of L-741,626 (D2A low, 1μg and D2A high, 10μg) and the corresponding vehicle (DMSO) on Fos expression in oxytocin cells in the SON of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.25A show the effect of DMSO, D2A low and D2A high on Fos expression in oxytocin cells in the SON of I males. Values are the group means ± SEM. Neither dose of the D2A (D2A low=1μg, n=3 and D2A high=10μg, n=6) had any significant effect on Fos expression in oxytocin cells in the SON of I males (One Way ANOVA) compared to vehicle (DMSO, n=6) (Figure 3.25B). Filled arrows show a Fos-positive oxytocin cell and empty arrows show a Fos-negative oxytocin cell. No effect between treatment groups was seen in the ALONE and NI males. Scale bars represent 100μm OX=optic chiasm.

Figure 3.26: Effect of the D3A and D4A on Fos expression in oxytocin neurons in the SON



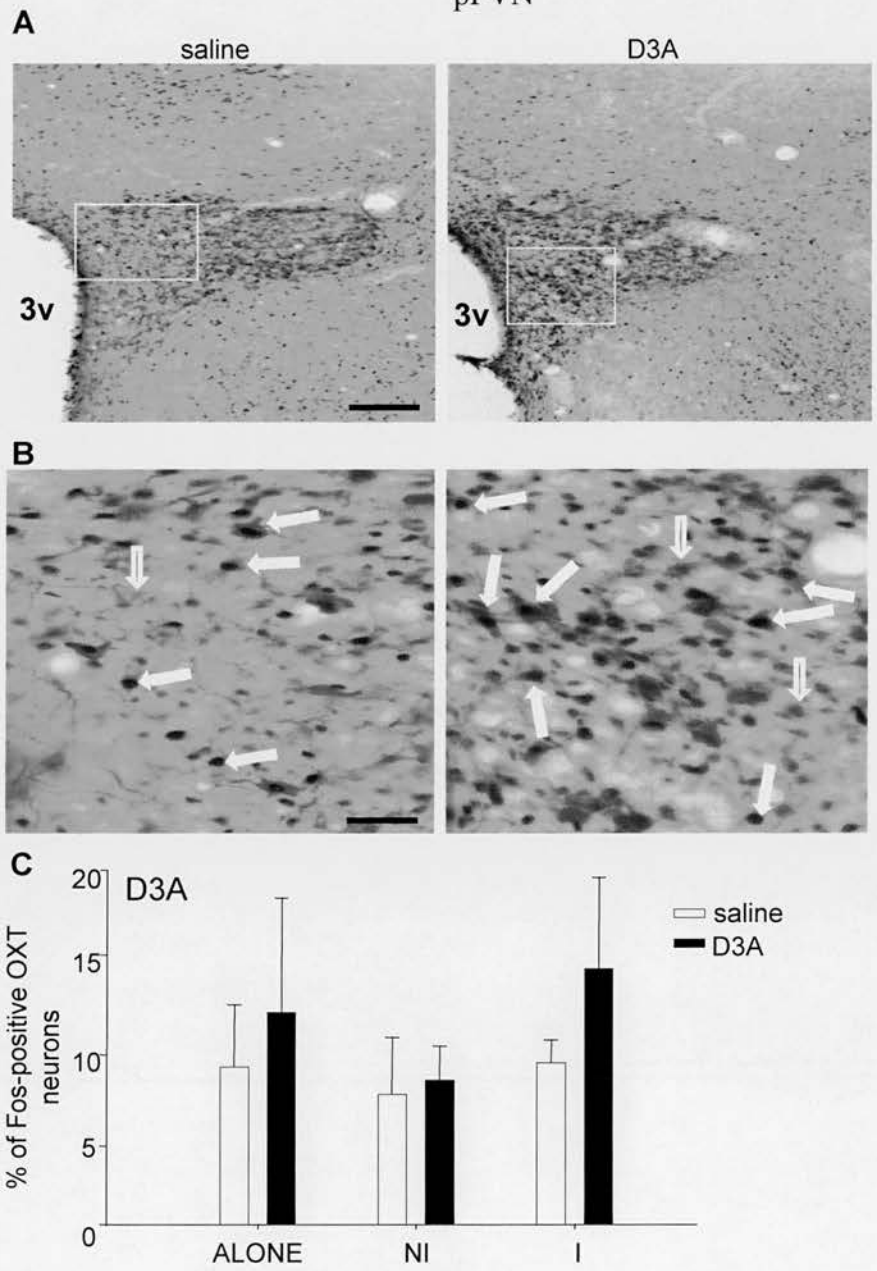
The effect of nafadotride (D3A, 5µg) and L-745, 870 (D4A , 7µg) and the corresponding vehicle (saline) on Fos expression in oxytocin cells in the SON of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.26A show the effect of saline, D3A and D4A on Fos expression in oxytocin cells in the SON of I males. Values are the group means ± SEM. The D4A (n=5) but D3A (n=5) significantly decreased the percentage of Fos-positive oxytocin cells expressed in the SON (Student's t-test, *P<0.05)of I) males compared to vehicle (saline, n=9) (Figure 3.26 B and 3.26C). Filled arrows show a Fos-positive oxytocin cell and empty arrows show a Fos-negative oxytocin cell. No effect between treatment groups was seen in the ALONE and NI males. Scale bars represent 100µm OX=optic chiasm.

Figure 3.27: Effect of the D2A on Fos expression in oxytocin neurons in the pPVN



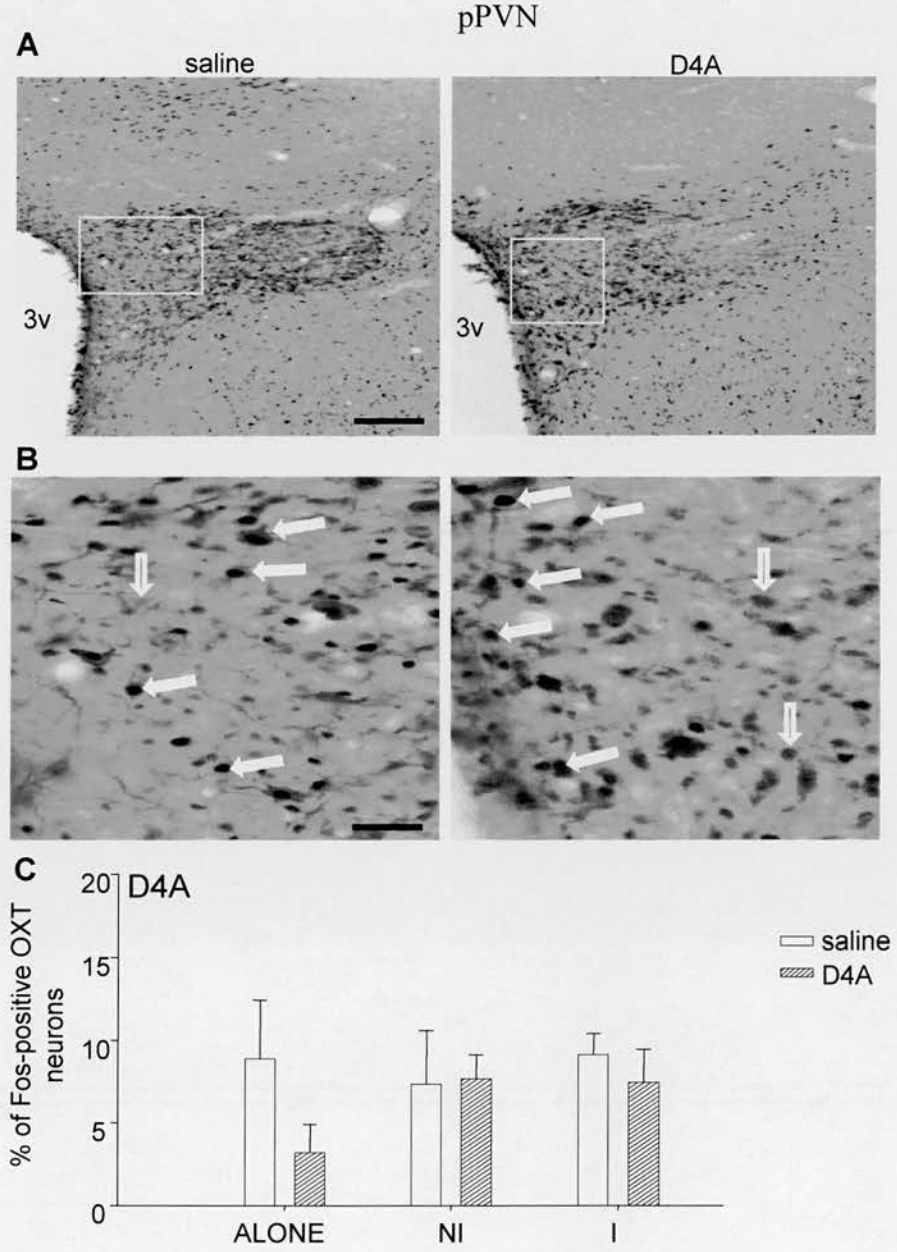
The effect of L-741,626 (D2A low, 1µg and D2A high, 10µg) and the corresponding vehicle (DMSO) on Fos expression in oxytocin cells in the pPVN of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.27A and 3.27B (magnification of highlighted areas in 3.27A) show the effect of DMSO, D2A low and D2A high on Fos expression in oxytocin cells in the pPVN of I males. Values are the group means ± SEM. Neither dose of the D2A (D2A low=1µg, n=3and D2A high=10µg, n=6) had any significant effect on Fos expression in oxytocin cells in the pPVN of I males (One Way ANOVA) compared to vehicle (DMSO, n=6) (Figure 3.27C). Filled arrows show a Fos-positive oxytocin cell and empty arrows show a Fos-negative oxytocin cell. No effect between treatment groups was seen in the ALONE and NI males. Scale bars represent 200µm and 50µm in 3.27A and B respectively. 3v=third ventricle.

Figure 3.28: Effect of the D3A on Fos expression in oxytocin neurons in the pPVN



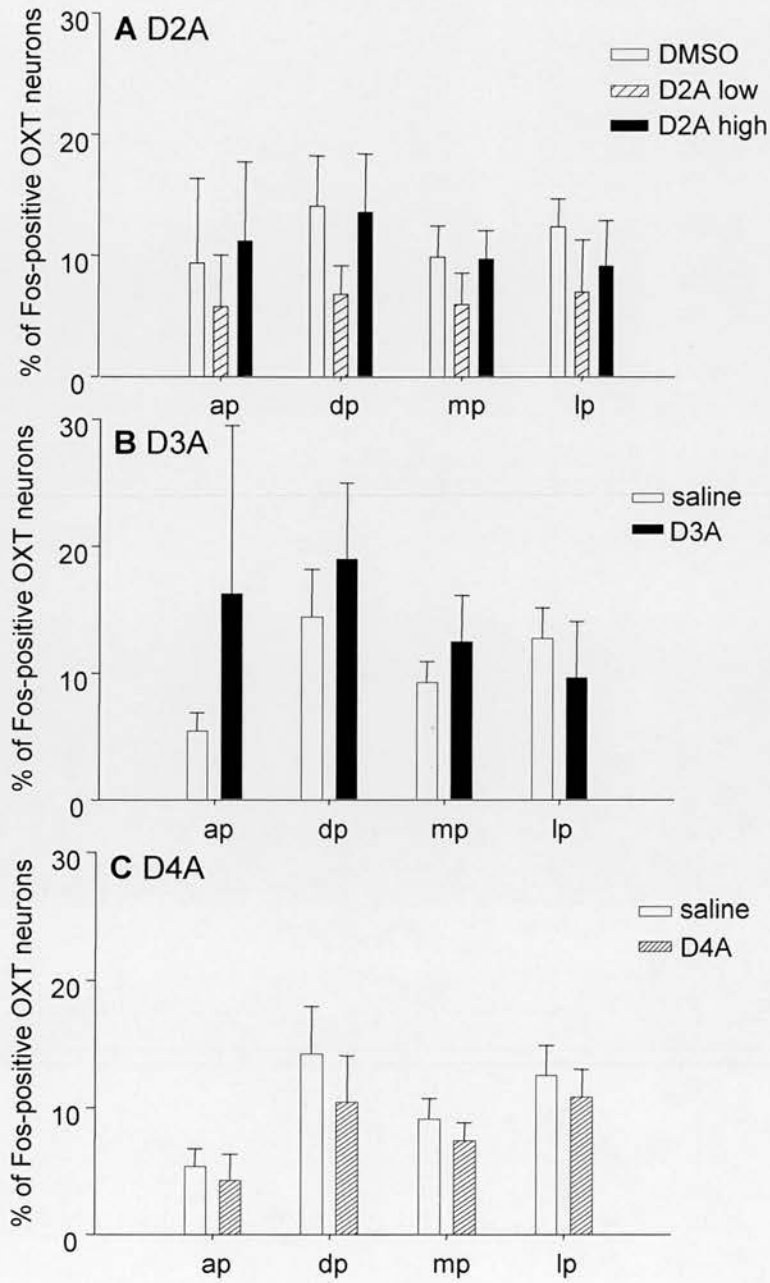
The effect of nafadotride (D3A , 5μg) and the corresponding vehicle (saline) on Fos expression in oxytocin cells in the pPVN of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.28A and 3.28B (magnification of highlighted areas in 3.28A) show the effect of saline and D3A on Fos expression in oxytocin cells in the pPVN of I males. Values are the group means ± SEM. The D3A (n=5) did not significantly decrease the percentage of Fos-positive oxytocin cells expressed in the pPVN of I males (Student's t-test) compared to vehicle (saline, n=9) (Figure 3.28C). Filled arrows show a Fos-positive oxytocin cell and empty arrows show a Fos-negative oxytocin cell. No effect between treatment groups was seen in the ALONE and NI males. Scale bars represent 200μm and 50μm in 3.28A and 3.28B respectively. 3v=third ventricle.

Figure 3.29: Effect of the D4A on Fos expression in oxytocin neurons in the pPVN



The effect of L-745, 870 (D4A , 7μg) and the corresponding vehicle (saline) on Fos expression in oxytocin cells in the pPVN of those males placed alone (ALONE), those who did not intromit (NI) and those who did intromit (I). Photomicrographs in Figure 3.29A and 3.29B (magnification of highlighted areas in 3.29A) show the effect of saline and D4A on Fos expression in oxytocin cells in the pPVN of I males. Values are the group means ± SEM. The D4A (n=5) did not significantly decrease the percentage of Fos-positive oxytocin cells expressed in the pPVN of I males (Student's t-test) compared to vehicle (saline, n=9) (Figure 3.29C). Filled arrows show a Fos-positive oxytocin cell and empty arrows show a Fos-negative oxytocin cell. No effect between treatment groups was seen in the ALONE and NI males. Scale bars represent 200μm and 50μm in 3.29A and B respectively. 3v=third ventricle.

Figure 3.30: Effect of the D2A, D3A and D4A on Fos expression in oxytocin neurons in the subdivisions of the pPVN



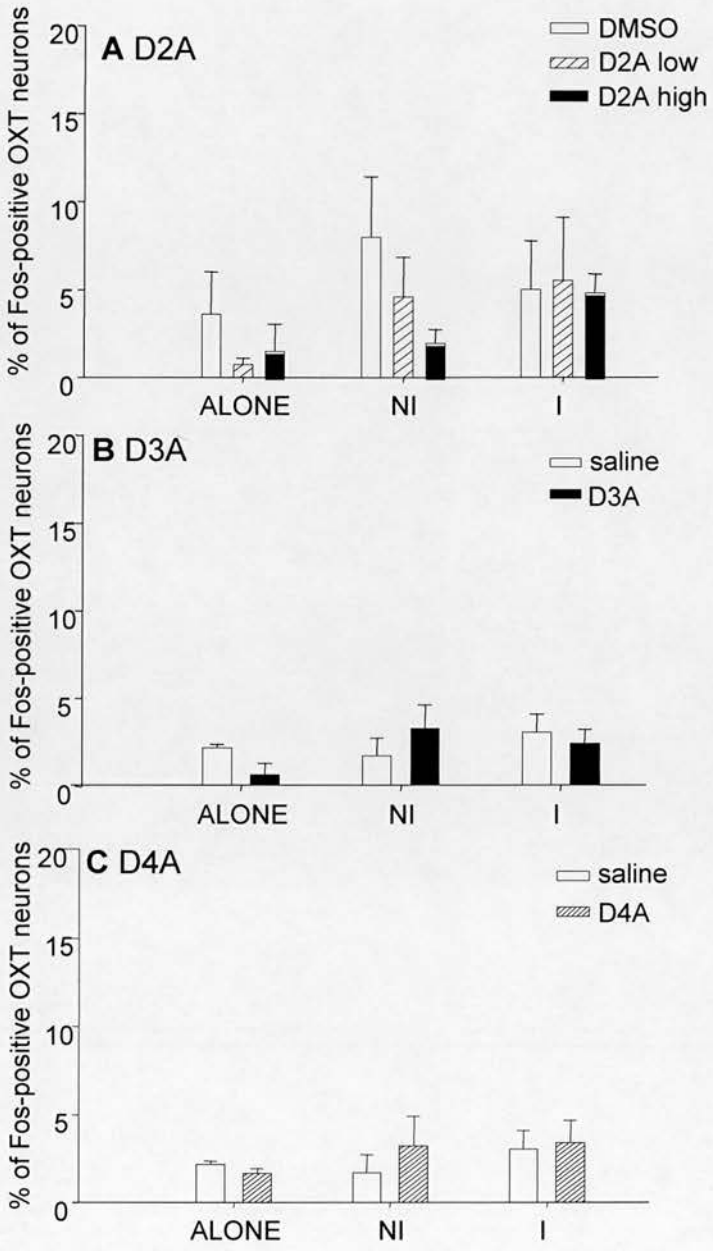
The effect of L-741,626 (D2A low, 1µg and D2A high, 10µg) (3.30A), nafadotride (D3A 5µg) (3.30B), L-745,870 (D4A 7 µg) (3.30C) and the corresponding vehicle (DMSO or saline) on Fos expression in oxytocin cells in the subdivisions of the PVN of those males those who intromitted(I). Values are the group means ± SEM. Neither the D2A (D2A low, n=3 or D2A high, n=6), D3A (n=5) or D4A (n=5) had any significant effect on Fos expression in oxytocin cells in the subdivisions of the PVN of I males (One Way ANOVA or Student's t-test) compared to corresponding vehicle (DMSO, n=6 or saline, n=9). No effect between treatment groups was seen in the ALONE and NI males. (data not shown). 3v=third ventricle; ap=anterior parvocellular; dp=dorsal parvocellular; mp=medial parvocellular; lp=lateral parvocellular.

D4A had any significant effect on Fos expression in oxytocin cells in any of the subdivisions of the PVN of I males (One Way ANOVA or Student's t-test) compared to corresponding vehicle (DMSO or saline). No differences were detected between treatment groups in the males placed alone (ALONE) or those who did not intromit (NI) (data not shown).

mPVN

Figure 3.31 displays the percentage of Fos-positive oxytocin cells in the mPVN after i.c.v. injection D2A (1µg or 10µg), D3A (5µg) or D4A (7µg) respectively of intromitting (I) males. Neither the D2A (D2A low or D2A high) (Figure 3.31A), D3A (Figure 3.31B) or D4A (Figure 3.31C) had any significant effect on Fos expression in oxytocin cells in the mPVN of I males (One Way ANOVA or Student's t-test) compared to corresponding vehicle (DMSO or saline). No differences were detected between treatment groups in the males placed alone (ALONE) or those who did not intromit (NI).

Figure 3.31: Effect of the D2A, D3A and D4A on Fos expression in oxytocin neurons in the mPVN



The effect of L-741,626 (D2A low, 1µg and D2A high, 10µg) (3.31A), nafadotride (D3A 5µg) (3.31B), L-745,870 (D4A 7 µg) (3.31C) and the corresponding vehicle (DMSO or saline) on Fos expression in oxytocin cells in the mPVN of those males those who intromit ted(I). Values are the group means ± SEM. Neither the D2A (D2A low, n=3 or D2A high, n=6), D3A (n=5) or D4A (n=5) had any significant effect on Fos expression in oxytocin cells in the mPVN of I males (One Way ANOVA or Student's t-test) compared to corresponding vehicle (DMSO, n=6 or saline, n=9). No effect between treatment groups was seen in the ALONE and NI males.

Chapter 3

3.4 Discussion

3.4.1 Effects of D2-like ligands on penile erection

3.4.1.1 Effects of D2-like agonists on penile erection

Both Quinelorane and PD168077 were potent stimulators of penile erection in naive conscious male rats. Those rats receiving the dopaminergic agonists did not display any stereotypic side-effects often associated with application of high doses of dopaminergic agents which include excessive sniffing, gnawing, frequent head bobbing and a locomotor stimulant response. Thus, the enhancement of penile erection episodes observed in this study was primarily due to the actions of dopaminergic agents on central erectile pathways and not due to adverse effects on those controlling locomotion.

3.4.1.2 Quinelorane (D2/D3 agonist) and penile erection

Quinelorane, a D2/D3 receptor agonist, was particularly effective in eliciting penile erection in the male rats. Quinelorane was chosen because it has been shown to have facilitative effects on rat copulatory behaviour (Foreman & Hall, 1987) and penile erections in rhesus monkeys (Pomerantz, 1991). When given into the PVN (Eaton et al, 1991), MPOA (and paired with physiological levels of testosterone) (Bazzett et al, 1991) or subcutaneously (Doherty et al, 1994), Quinelorane strongly induces penile erections and decreases the latency to the first penile erection (Eaton et al, 1991). Quinelorane has also been known to have a biphasic effect with low to moderate doses (1μg) facilitating penile erection and high doses (10μg) inhibiting erectile function and facilitating seminal emission in the supine male rat (Bitran et al, 1989; Eaton et al, 1991). Thus, in this study, a 1μg dose of Quinelorane was given i.c.v to examine the effects on penile erection in the isolated conscious male rat.

Consistent with previous studies, Quinelorane showed clear pro-erectile effects in conscious male rats by increasing the percentage number of rats exhibiting erections and also the mean number of erections per rat. Additionally, Quinelorane was equally effective in eliciting stretching/yawning

behaviour in 80-90% of the rats. Such an effect has been previously reported (Docherty and Wisler, 1994) and can be attributed to the relative non-selectivity of Quinelorane at the D2/D3 receptors. However, it is unclear as to which receptor (D2 and/or D3) mediates the pro-erectile effects of Quinelorane. It is believed that this dual behavioural effect is due to activation of D2 receptors in the stimulation of penile erection and activation of D3 receptors in eliciting stretching/yawning. However, it has proved very difficult to establish an in vivo model that will discriminate between D2 and D3 receptor-mediated activities, primarily due to the lack of commercially available highly selective D2-like receptor agonists. Quinelorane-induced penile erection and yawning are inhibited by the D2-preferring antagonist, haloperidol (Docherty and Wisler, 1994) and several D3-preferring receptor antagonists (Collins et al, 2005). Additionally, the D3 receptor agonist, 7-OH-DPAT can also facilitate penile erection (Ferrari and Giuliani, 1995). Thus, it seems that both receptors have a modulatory role at least, in mediating penile erection and stretching/yawning, although it is not clear whether there is reciprocal or sequential involvement of D2 and D3 receptors. Interestingly, it is understood that in any population of hypothalamic D2-like receptors, the D2 receptor type is more abundantly expressed than D3 and D4 receptors (Khan et al, 1998). Thus perhaps proportionately, more D2 receptors will be activated compared to D3 or D4 receptors. So, we can speculate that the pro-erectile effect of Quinelorane is likely to be due in part to preferential activation of central D2 receptors.

3.4.1.3 PD168077 (D4 agonist)

PD168077 is a highly selective D4 receptor agonist which acts as a partial agonist in-vitro (Gazi et al, 2000). PD168077 has strong pro-erectile effects when given subcutaneously, i.c.v., or intra-PVN (Hsieh et al, 2004; Melis et al, 2005; Melis et al, 2006). In this study, as expected, when given i.c.v., PD168077 significantly increased the % of rats displaying erection as seen in previous studies (Hsieh et al, 2004; Melis et al, 2006) and non-significantly increased the number of erections per rat compared to vehicle. Only 60% of rats receiving PD168077 displayed penile erection, while other studies found 80% of PD168077-treated rats showed erection (Hsieh et al, 2004), despite similar doses being used. An explanation for this may be due to the different drug volumes applied (2µl in our study versus 5µl in Hsieh et al, 2005) or alternatively due to the physical environment where the

observations were made. We did not see any spontaneous erections in the vehicle-treated group which was surprising as the literature generally indicates that at least 10% of rats are expected to display approximately 0.8-1 penile erection episode per rat within a 60 min observation period. However, during our experiment, penile erection episodes were observed under a normal light cycle (lights on 07:00am-7:00pm), approximately 4 hours in to the light period, as opposed to during the dark period as seen by Hsieh and colleagues. It is well known that rats will generally only display copulatory behaviour during the second phase of the dark period. Therefore, our vehicle-treated and perhaps drug-treated rats may have elicited more erectile episodes if placed in the dark prior to and during the test period.

3.4.1.4. Effects of D2-like antagonists on intromission

D2A

Rats receiving the dopaminergic antagonists did not show any apparent deficits in sniffing, gnawing or locomotor behaviours. Thus, the reduction in the expression of penile erection episodes observed in this study was primarily due to the actions of dopaminergic agents partly blocking central erectile pathways and not due to adverse effects on those controlling locomotor behaviour.

We investigated the effects of the D2-like antagonists, L-741,626 (D2A), nafadotride (D3A) and L-745,870 (D4A) on intromission in males placed with receptive females. During copulation, neither dose of the D2A (1μg and 10μg) sufficiently inhibited intromitting behaviour. Both a low and a high dose of D2A were separately administered because previous studies have shown dopaminergic ligands such as Quinelorane and apomorphine to have biphasic effects where they facilitate penile erection at low doses and inhibit penile erection at high doses (Bitran et al, 1989; Eaton et al, 1991; Hull et al, 1992). Thus, to pharmacologically confirm this, we gave low and high doses of the D2A to see if they could inhibit and facilitate penile erection, respectively. Unfortunately, only 6/14 rats in the corresponding vehicle-treated group (isotonic saline with DMSO comprising 20% of overall volume) for the D2A displayed intromission which was unexpectedly low, as normally approximately 80% of rats in the control group should display full copulatory behaviour. This impairment in copulatory behaviour after central administration of compounds containing DMSO has been reported once before

by Bitner and colleagues (2006). They administered PD168077 dissolved in 100% DMSO and found that none of their PD168077-treated rats displayed any penile erection (Bitner et al, 2006). The reason for this remains unclear; however, it seems logical to assume that DMSO may somehow interrupt central neural pathways and/or intracellular signalling pathways that mediate penile erection. This may be due to general cellular toxicity effects of DMSO or it may be a behaviourally-specific effect (eg. penile erection). Conversely, other studies that have used DMSO to dissolve their drug have shown no apparent detrimental effects in regard to the pro-erectile activity of the drug (Melis et al, 2005; Melis et al, 2006; Succu et al, 2007). However, the experimental design was different in these studies since Melis and colleagues studied penile erection in conscious isolated males. Perhaps, DMSO interferes with social and olfactory cues which would affect the male when pairing with a receptive female (as opposed to affecting a socially isolated male) and therefore impact on sexual arousal. This seems unlikely however, as all vehicle-treated and most of the D2A-treated males displayed mounting behaviour and anogenital investigation. Therefore, perhaps it is the integration of olfaction with the stimulation of central pathways that regulate sexual arousal that is affected by DMSO.

Because the vehicle-treated rats performed poorly, this finding makes it very difficult to interpret the effects of the D2A (both low and high doses) on intromission. With the low dose of D2A only 30% of rats exhibited intromission whilst 50% (similar to vehicle) of rats were able to intromit with the high dose of D2A, thus it is tempting to suggest that the D2A may act in biphasic manner. Further studies are needed to confirm this.

D3A

D3 receptors have recently been implicated in central control of ejaculation in anaesthetised rats (Clement et al, 2007; Kitrey et al, 2007); however, it is not known if blockade of central D3 receptors impacts on penile erection in males placed in a mating environment. Prior to this study and after an extensive literature search, it was apparent that nafadotride had not been injected i.c.v. previously, so it was unclear as to which dose to administer. From studies administering nafadotride i.p., an approximate estimation of a central dose was calculated. Here, we showed that the D3A (nafadotride,

5μg) inhibited the percentage of rats achieving intromission (which was almost significant), yet had no effect on any other copulatory parameters. Thus, blockade of central D3 receptors appears to affect the pro-erectile effects of endogenous dopamine. Expression of central D3 receptors is comparatively scarce compared to D2 and D4 receptor expression. Thus, it seems unlikely that D3 receptors primarily mediate penile erection induced by endogenous dopamine. However, it can be said that D3 receptors may play a minor role in the expression of erectile function.

D4A

Due to the recent (particularly since 2000) introduction of highly selective D4 receptor pharmacological ligands, studies on the role of D4 receptors during penile erection have proved most convincing (Melis et al, 2005; Melis et al, 2006; Succu et al, 2007). The effect of i.c.v. injection of the D4A (L-745,870) on penile erection had not been published prior to this study, although it had been administered unilaterally into the PVN at a dose of 1μg. Thus, as with the D3A, an approximate estimation of an i.c.v dose was calculated, which was slightly higher than that of the intra-PVN dose in order to compensate for dilution of the D4A in the CSF and distribution to areas other than the three hypothalamic nuclei of interest. In our study, the D4A (L-745,870, 7μg) proved to be the most effective of the three antagonists used in inhibiting intromission and also increased the length of time for mounting behaviour to be displayed. This increase in mount latency after D4A injection lightly touches on a possible role for the D4 receptor in sexual arousal in the rat as recent evidence suggests in humans (Ben Zion et al, 2006). The inhibitory effect of the D4A during copulation has not been reported before. Here we show that whilst the D4A inhibited the incidence of intromission, it did not affect intromission latency or frequency which suggests that D4 receptor activation is involved in the initiation and achievement of penile erection. However, sexual events (eg. frequency of intromissions, pelvic thrusting) occurring after penile erection may be modulated via other dopamine receptors and/or additional direct/indirect pathways. Thus, central D4 receptors appear to be important in mediating the effects of endogenous dopamine during penile erection in a physiologically sexually exciting environment. Moreover, for the first time, these data tentatively indicate that central D4 receptors may also be involved in the more appetitive phases of sexual behaviour.

3.4.2 Effects of D2-like ligands on neuronal activation in the MPN, SON and PVN

It has been shown that dopaminergic ligands acting on hypothalamic nuclei can alter oxytocin release (Bridges et al, 1976) and so it has been suggested that dopaminergic agonists and antagonists that facilitate and inhibit penile erection, respectively, may do so via acting on hypothalamic oxytocin neurons (Melis et al, 1989a, 1989b, 1990). As the SON, PVN, and to a lesser extent the MPN, are rich in oxytocin, and Fos is an accepted marker of neuronal activation, we performed single Fos and double Fos and oxytocin immunocytochemistry in rats treated with a range of pharmacological tools that can mimic or block the effects of endogenous dopamine.

3.4.2.1 Effects of Quinelorane and PD168077 on neuronal activation

Rats receiving Quinelorane (D2/D3 receptor agonist) but not PD168077 (D4 receptor agonist) showed increased Fos expression in the pPVN (particularly the medial parvocellular subdivision) but neither drug had any effect in the MPN, SON or mPVN. Likewise, Quinelorane but not PD168077 increased the percentage of oxytocin cells expressing Fos in the pPVN (particularly the medial parvocellular subdivision), indiating that D2/D3 receptor stimulation activates parvocellular oxytocin neurons. The apparent lack of effect on magnocellular oxytocin neurons may be unexpected, since Quinelorane has been shown to act on mPVN neurons to stimulate peripheral release of oxytocin in male rhesus monkeys (Amico et al, 1993). However, here we show that during penile erection, Quinelorane selectively activates parvocellular oxytocin neurons. The pPVN is comprised of different subregions, these include: anterior, dosal, medial and lateral parvocellular subdivisions (ap, dp, mp and lp). Oxytocin neurons in some of these regions (namely, the dorsal, medial and lateral parvocellular regions) project to the spinal cord (Hallbeck et al, 2001; Swanson & Sawchenko, 1980; Wagner et al, 1993), and synapse onto parasympathetic nuclei in the lumbosacral spinal cord to stimulate autonomic functions such as penile erection in males (Veronneau-Longueville et al, 1999; Tang et al, 1998) and uterine contractions in the female rat (Benoussaidh et al, 2005). It has been suggested there might be a pPVN subregion(s) specifically activated during male sexual behaviour. The lateral parvocellular oxytocin cells have been shown to be activated in copulating male rats (Witt and Insel, 1994).

Additionally, penile neuronal nitric oxide synthase (PnNOS), a stimulatory protein required for erection, was found to be expressed in the medial parvocellular regions of the PVN (Ferrini et al, 2003); although it is not known if pnNOS was found to be co-localised with oxytocin.

3.4.2.2 Quinelorane and oxytocin neuronal activation

We show that medial parvocellular oxytocin neurons are activated by Quinelorane (almost significant effects were observed in the lateral parvocellular region too, P=0.08) which adds to some previous studies implicating the selective involvement of pPVN regions in the mediation of penile erection (Kita et al, 2006; Witt and Insel, 1994). Kita and colleagues previously showed that the dorsal, medial and lateral parvocellular subregions which all project to the spinal cord, were shown to be activated during penile erection after i.c.v injection of oxytocin (Kita et al, 2006). It is of interest that although the Quinelorane-treated rats expressed more penile erection episodes, they also displayed greater yawning behaviour when compared with vehicle-treated rats. Yawning and penile erection have both been shown to involve activation of medial parvocellular oxytocin neurons (Kita et al, 2006), thus it is not entirely clear if the increased activation is associated with yawning and/or penile erection. It does appear to be partly due to penile erection as when superficially comparing those vehicle- and Quinelorane-treated males displaying yawning behaviour only, no significant differences in oxytocinergic activation were observed. Because dopaminergic agents like Quinelorane are potent inducers of stretching-yawning behaviour as well as penile erection (such stretching-yawning effects have not previously be seen with PD168077), care had to be exercised when examining Fos induction after Quinelorane and PD168077 administration. After quantifying stretching-yawning-induced Fos expression only, after each drug treatment, it could be seen that there were no significant differences between vehicle, Quinelorane and PD168077 (although "n" numbers were very low, approximately 1-3) (data not shown). However, when comparing Fos expression with vehicle –treated rats that exhibited stretching-yawning but not penile erection with Quinelorane-treated rats that expressed stretching-yawning and penile erection, significant differences in Fos expression were observed (no significance was observed in PD168077-treated rats). Thus, the significant induction of Fos expression in Quinelorane-treated rats compared to vehicle-treated rats is primarily due to the pro-erectile effects of Quinelorane and not the accompanying stretching-yawning effects. Another

possible reason as to why we saw an increased expression of Fos-positive oxytocin cells in the medial parvocellular area in particular is that this subregion of the PVN receives a significantly dense dopaminergic innervation compared to the other subregions of the pPVN (Swanson et al, 1981). Thus, with such an anatomical arrangement it seems logical to assume that the medial parvocellular oxytocin cells play some role in mediating the pro-erectile effects of Quinelorane.

Therefore, we speculate that medial (and perhaps lateral) parvocellular oxytocin neurons originating in the PVN and projecting to the spinal cord, mediate the pro-erectile effect of Quinelorane. It is has been demonstrated, that descending paraventriculospinal oxytocin pathways may have more of a modulatory rather than primary role in the control of penile erection. Indeed, paraventricular lesions destroying descending projections significantly reduced apomorphine and oxytocin-induced penile responses (Argiolas et al, 1987). However, it has also been demonstrated that selective chemical lesions of the parvocellular neurons do not significantly affect copulation but can reduce seminal plug weights (Ackerman et al 1997), increase the latency and reduce the number of non-contact erections (NCE) (Liu et al, 1997). Thus, overall copulatory function is not significantly impaired and only selected parameters specific to penile erection seem to be affected. In the lesion studies, the apparent lack of effect on copulation could be attributed to increased peripheral involvement and / or the presence of genital stimulation during coitus which may subsequently overcome the spinal oxytocin deficits induced by PVN lesions.

Thus, it seems spinally-projecting oxytocinergic fibres may primarily have a permissive role in penile erection and perhaps a relatively minor role in copulation. During penile erection, it seems likely that other key central neuromodulators have an equally important role. Dopaminergic agonists acting in the lumbosacral spinal cord have also been shown to have a strong facilitative effects on penile erection (Guiliano et al, 2001) and spinally-projecting serotonergic fibres may also contribute to central control of penile erection (Bancila et al, 1999) and facilitate ejaculatory parameters (Stafford et al, 2006). Thus, it would appear that achievement of penile erection is a summative effect of activation of multiple neural substrates including central dopaminergic, oxytocinergic and serotonergic pathways.

3.4.2.3 PD168077 and oxytocin neuronal activation

Although the D4 receptor agonist, PD168077 was clearly pro-erectile, it was not as potent as the D2/D3 receptor agonist, Quinelorane, and did not appear to have any significant effect on Fos or combined Fos and oxytocin expression in the MPN, SON or PVN. This was surprising as PD168077 has previously been shown to induce Fos expression in the PVN (Bitner et al, 2006). However, that study did not record penile erection and the neuronal phenotype expressing Fos protein was not reported (Bitner et al, 2006). Additionally, different routes of administration were applied (subcutaneous versus i.c.v. in our study) which could also have affected the outcome of the results. It is apparent that PD168077 elicits penile erection in the conscious rat but not via hypothalamic oxytocin neurons, suggesting D4 receptor activation stimulates alternative or indirect pathways. These findings are based on administration of single doses, ideally full dose response curves should be constructed to further confirm the effects of Quinelorane and PD168077 on the activation of oxytocin neurons.

One such pathway may involve hypothalamic GABAergic fibres which may serve as a potential indirect pathway in the expression of penile erection. It is known that a complex cellular hierarchy exists in the SON and PVN whereby oxytocin neurons are heavily modulated by inhibitory GABAergic and excitatory glutamatergic neurons which themselves can be influenced by a range of synaptic inputs and neurotransmitters. GABAergic and glutamatergic neurons synapse with magnocellular oxytocin soma and dendrites in the PVN and SON whereas dopaminergic innervation of these nuclei is comparatively scarce (Jourdain et al, 1999). Thus, it seems reasonable to assume that endogenous dopamine could exert it's effect on oxytocin release by acting indirectly via glutamatergic and/or GABAergic neurons rather than via oxytocin neurons directly. Evidence for an intermediary role for GABA includes that intra-PVN injection of muscimol, the $GABA_A$ receptor agonist, inhibits apomorphine- and oxytocin-induced penile erections (Melis and Argiolas, 2002). In particular, the D4 receptor has been implicated in the disinhibition of GABAergic and inhibition of glutamatergic neurotransmission in the SON by acting presynaptically (Price and Pittman, 2001; Azdad et al, 2003). Although, it is unclear as to which system would predominate under physiological

conditions. It could be speculated that dopamine-induced inhibition of GABAergic and glutamatergic neurons would somehow increase the responsiveness of the postsynaptic cell, conferring on it a greater sensitivity, free to respond to other excitatory factors such as oxytocin. So, it could be that during penile erection, dopamine acts presynaptically to disinhibit oxytocin neurons and so facilitate the activation of the hypothalamic oxytocinergic system.

From the present study, it appears that the D2/D3-preferring agonist, Quinelorane, is a potent inducer of penile erection and may stimulate penile erection by acting on pPVN oxytocin cells but not those in the MPN and SON. Very little is known regarding the role of the SON during sexual behaviour. Supraoptic oxytocin cells have been implicated in intromission and ejaculation (Caquineau at al, 2006; Pattij et al, 2005). In the penile erection study, we looked at the effects of a dopamine agonist on penile erection only in sexually naive male rats placed individually in cages in a male only experimental room and there were no females rats present. Thus, our males had not intromitted or ejaculated and had not even been exposed to female oestrus cues which may explain the lack of effect on magnocellular oxytocin neurons. Perhaps a stronger environmental and/or physiological stimulus is required for dopamine agonists to activate magnocellular oxytocin cells or perhaps these neurons are only activated upon intromission and ejaculation.

Likewise, even less is known regarding oxytocin cells in the MPN in the sexual behaviour context. The MPN is part of the MPOA, in addition to being highly sensitive to the dopaminergic agonist, apomorphine, it is fundamentally important in the control of male copulatory function (Paredes and Agmo, 2004 for review). However, to our knowledge, there are no data examining the effect of dopaminergic agonists on oxytocin neurons within the MPN during penile erection in the rat. Here, we show that penile erection elicited by Quinelorane and PD168077 does not involve the activation of MPN oxytocin cells, suggesting that dopamine-receptor mediated penile erection in the MPN is mediated via another neuronal phenotype(s). Alternatively, the MPN is not a particularly important integration site for dopamine-mediated penile erection yet crucial for dopamine-mediated copulation as previously reported.

Thus, both Quinelorane and PD168077 have a facilitatory effect on erectile function in the conscious male rat. Only Quinelorane, however, appeared to significantly activate oxytocin neurons in pPVN and was without effect in the MPN, SON and mPVN. Therefore it could be postulated that activation of D2-like receptors stimulates penile erection via acting on oxytocinergic pathways, dependent upon the receptor subtype and selected neuronal populations in specific hypothalamic nuclei. However, non-oxytocinergic pathways cannot be ruled out.

3.4.2.4 Effects of D2-like antagonists on intromission-induced neuronal activation

D2A and oxytocin neuronal activation

The D2A (both 1μg and 10μg) did not affect Fos or double labelled Fos and oxytocin expression in the MPN, SON or PVN during intromission, suggesting that hypothalamic oxytocin neurons do not mediate D2-receptor mediated penile erection in a sexually exciting environment. As previously stated, the D2A data must be interpreted with caution due to the unexpectedly low number of control males displaying mounting and intromitting behaviour. The low dose of the D2A had a marginally inhibitory effect in the MPN, SON and PVN but the high dose of the D2A did not have any significant effect in any of the three brain nuclei. However, a significant increase in Fos expression after the high dose of D2A was seen in the anterior parvocellular subdivision of the pPVN. The reason for this is unclear, but perhaps at such high doses the D2A is acting at dopamine autoreceptors and so acts to increase dopamine concentrations in the synaptic cleft. Or as with the biphasic effects of the agonists, apomorphine and Quinelorane, the D2A also has a biphasic effect with high doses facilitating penile erection. This is unlikely however, as all rats in the D2A groups displayed poor copulatory performance, possibly due to DMSO as discussed earlier. Finally, it is known that the D2-preferring antagonist, haloperidol induces *c-fos* mRNA and Fos protein expression in the striatum and nucleus accumbens, however, this was not observed in a sexual behaviour context (Nguyen et al, 1992; Rogue and Vincendon, 1992). This Fos inducing effect has not been shown for L-741,626, however, it may help to explain the lack of an effect of the low dose of D2A on intromission-induced Fos and combined Fos and oxytocin expression.

D3A and oxytocin neuronal activation

The most surprising finding of this study was the ability of the D3A (nafadotride) to inhibit intromission-induced Fos expression in the MPN, SON and PVN, yet it did not significantly inhibit the percentage of oxytocin cells expressing Fos in these three nuclei. For the first time, we have shown that the D3A inhibits certain copulatory parameters in conscious male rats, however, it does not appear to act through the hypothalamic oxytocinergic system but via other unknown hypothalamic neuronal phenotype(s). With oxytocin as the exception, very little is known regarding the intra-hypothalamic neural circuitries that facilitate male sexual behaviour. The MPN, SON and PVN are nuclei that are separately involved in many various and specific neuroendocrine-mediated behaviours and so each express a heterogenous cellular population. Thus, in our study it is very difficult to postulate which particular cell phenotype(s) within the nuclei displayed attenuated Fos expression after administration of the D3A. In the MPN gonadal steroid-dependent galanin and GnRH neurons have both been implicated in the regulation of sexual behaviour and could serve as potential target neurons for endogenous dopamine (Dennison et al, 1996; Bloch et al, 1993; Bloch et al, 1998). In the PVN, NMDA and the melanocortin, α-MSH, are both believed to enhance consummatory aspects of male sexual behaviour (Caquineau et al, 2006; Giuliano 2004; Melis et al, 1994; Melis et al, 2004). However, it is not known if glutamatergic or A-MSH-containing neurons express dopamine receptors or whether dopamine modulates these neurons via indirect methods, for example via the hypothalamic endocannabinoid system. It has recently been shown that blockade of the cannabinoid CB1 receptors in the PVN stimulates penile erection (Castelli et al, 2007); an effect which occurs concomitantly to an increase in glutamic acid (a precursor for glutamate) concentrations in the PVN (Melis et al, 2006; Succu et al, 2006). Thus, endocannabinoids may mediate penile erection via the modulation of GABAergic and/or glutamatergic transmission in the PVN. The SON neurons generally express oxytocin and vasopressin and no other neuronal populations in the SON have been shown to be involved in sexual behaviour as yet, although, they co-express many other neuropeptides. A possible explanation for the attenuated Fos expression we observed in the SON, may be that D3A indirectly inhibits the activation of oxytocin neurons via acting on glutamatergic neurons that normally provide excitatory input to supraoptic oxytocin cells. The D3A may inhibit glutamatergic neurotransmission and subsequently oxytocin activity in the SON, although this is highly speculative.

D4A and oxytocin neuronal activation

The D4A (L-745,870) did not inhibit intromission-induced Fos expression in the hypothalamic regions analysed but it did significantly attenuate the percentage of Fos-expressing oxytocin cells in the SON but not the MPN or PVN. Therefore, we show that D4-receptor mediated intromission is mediated (in part) by supraoptic oxytocin neurons. This finding contrasts with our penile erection study, where the D4 receptor agonist, PD168077 did not appear to activate supraoptic oxytocin cells during penile erection. It is important to note that the two experiments were designed differently however. In this study, the rat was placed with a receptive female and allowed to copulate (as opposed to the D4 agonist study where the rat was in isolation). Thus perhaps such olfactory and chemosensory stimuli in addition to intromission (as seen in the intromission study) are required for the D4A to have an inhibitory effect on the activation of oxytocin cells in the SON as opposed to D4-receptor stimulation alone, in isolated males (D4 agonist study). It is interesting to state that in all studies which have implicated the SON in male sexual behaviour, all rats had at least intromitted and most had ejaculated (Caquineau et al, 2006; Pattij et al, 2005; Philips-Farfan et al, 2007). Thus, it seems that D4-receptor mediated penile erection alone is not sufficient to activate oxytocin neurons in the SON. As to how L-745,870 inhibits the activity of oxytocin neurons is unclear. PD168077 (D4 agonist) and L-745,870 (D4A) have been shown to presynaptically inhibit and disinhibit, respectively, GABAergic neuron activity in the SON (Azdad et al, 2003). So as briefly described before, it is likely that L-745,870 acts presynaptically on GABAergic neurons to enhance GABAergic transmission and inhibit oxytocin neuron activity. Alternatively, if oxytocin cells in the SON express D4 receptors (investigated in chapter 4), L-745,870 may act directly on oxytocin cells to produce an inhibitory effect.

As expected each of the D2-like antagonists did not have any effect on neuronal activation in the non-intromitting males (those displaying only anogenital investigation and mounting behaviour). However, the males placed alone (in a novel environment without a female or female olfactory cues) showed relatively high basal levels of Fos and combined Fos and oxytocin expression which was unexpected. Perhaps the placing of the rat in a new environment has a greater effect on the stress

response than previously anticipated. Additionally, the same novel environment (eg. a freshly cleaned cage) was used for all the rats in the experimental groups so perhaps pheromones still present in the cage from the previously vacated male were sufficient to stimulate neuronal activation in the hypothalamus. Finally, in our intromission study, the male rat was placed in the receptive female's cage, whereas other studies have introduced the female into the male's cage (Dominguez et al, 2001; Lumley and Cooleen, 1999; Muschamp et al 2007; Olivier et al, 2007). Also, each ovariectomised receptive female was used 2-3 times for the copulatory tests. Some of these males displayed exploratory behaviour as opposed to sexual-associated behaviours and they expressed, what seemed to be, elevated aggressive behaviours (pinning down of the female, increased lengths of time in the dominant-subordinate position) which may be due to the presence of pheromones in the test cage from the previously vacated male. Thus, these factors may have contributed to the lower than average copulatory performance, elevated basal levels of neuronal activation and apparent lack of effect on neuronal activation of some of the dopamine antagonists.

In these two studies all drugs were given into the left lateral ventricle, so it is not known where each drug acted or at what concentration. Perhaps by injecting more locally into the MPN, SON or PVN itself we may have seen a stronger effect of the pharmacological ligands on sexual behaviour, Fos expression and combined Fos and oxytocin expression. This approach was not used since intra-nuclear injection would have damaged the tissue integrity of each nucleus and made accurate analysis of Fos and oxytocin expression very difficult. Perhaps a unilateral injection into each brain region may solve this problem and allow analysis of protein expression in the contralateral half of each nuclei. Although, it is known that such protein expression on one side of the nucleus does not always clearly reflect that of the other side or brain region as a whole.

Although Fos immunocytochemistry is a widely used technique enabling the tracing of neuroanatomical pathways involved in sexual behaviour, some researchers remain sceptical about the functional significance of Fos protein when investigating the direct effects of drugs. It is generically known as a marker of functional neuronal activation because it is believed to lead to long term changes in cell structure and function. However, this statement is ambiguous because it is not known

if Fos expression is indicative of for example, neuropeptide release, an influx of calcium or is indeed a direct measure of neuronal activation. Regarding sexual behaviour, Fos induction can be associated with various sensory stimuli and the activation of skeletal motor outputs required for copulation. Finally, the induction of Fos may serve as a "neuronal predictor" for the activation of certain brain nuclei known to be involved in the integration of future neuroendocrine-mediated behaviours such as sexual satiety. So, it seems that whilst Fos immunocytochemistry is an extremely useful investigative tool, its correlation to behavioural data must be interpreted with caution. Application of dopaminergic ligands to isolated oxytocin cells (for example using electrophysiology) and examining their effects on the intracellular calcium signal in oxytocin cells would perhaps provide a better measure of a direct interaction between dopamine and oxytocin pathways.

Thus, in conclusion, it is apparent that central dopamine D2-like receptors are important in the expression of penile erection in the male rat. However, the complex neural circuitries which subserve such dopamine-mediated sexual behaviour remain to be elucidated. The hypothalamic oxytocinergic system is one potential neuronal candidate that may mediate the excitatory and inhibitory effects of dopaminergic agonists and antagonists. The involvement of central D4 receptors during *in-copula* penile erection seems to be more important than that of D2 receptors which evidently has at most, a minor role. There is however, growing interest in the role of D3 receptors during erectile function and its underlying neural pathways. We provide evidence that D4 and D3 but not D2 receptors partly mediate penile erection via oxytocinergic pathways; but we cannot rule out involvement of non-oxytocinergic pathways (in the MPN, SON and PVN) as these are almost certainly also recruited.

In our dopamine agonist study, we have shown that during penile erection, the D2/D3 agonist, Quinelorane acts via medial parvocellular oxytocin neurons which are known to comprise a group of spinally-projecting fibres. Additionally, in our antagonist study we demonstrated that intromission and the concomitant activation of oxytocin cells in the SON, were antagonised by the D4A (L-745, 870). Thus, it is clear that dopaminergic ligands can modulate central oxytocinergic transmission; however, whether endogenous dopamine acts directly via oxytocin cells or indirectly during penile erection remains unknown. We have shown that dopaminergic ligands can augment oxytocin neuron

activity during penile erection and intromission. Therefore, it is possible to assume the involvement of a direct mechanism whereby hypothalamic oxytocin cells express D2-like receptors and so mediate the pro-erectile effects of dopamine. Thus, in the next chapter we set out to investigate the expression of D2, D3 and D4 receptors on oxytocin cells in the MPN, SON and PVN of sexually experienced male rats.

Chapter 4

Dopamine D2-like receptor and oxytocin colocalisation

Chapter 4

4.1 Dopamine D2, D3, D4 receptor and oxytocin expression in the MPN, SON and PVN

The actions of dopamine are modulated by the widespread D1-like and D2-like receptor subtypes. D1-like receptors classically act via positive coupling to adenylate cyclase, conversely, the D2-like receptors act via negative coupling to adenylate cyclase or through other signalling pathways such as MAP kinase (Bitner et al, 2004). Both receptor subgroups functionally interact in the brain, whereby activation of one receptor subclass can affect the function of another, such as seen in the striatum (Harsing and Zigmond, 1997) and nucleus accumbens (Moine and Bloch, 1996). Dopamine receptor activation can induce the stimulation of various transduction pathways; however, this is highly dependent on the nuclear and cellular location as well as the cell-specific G-protein coupled transduction pathways to which each receptor is associated with.

Dopamine receptor expression and distribution in the rat brain is widely known (Missale et al, 1998; Sokoloff and Schwartz for review, 1995). D1 receptor expression in the CNS is most abundant followed by D2 receptor expression (Ariano et al, 1989; Mansour et al 1990; Missale at al, 1998 for review). D3, D4 and D5 receptor expression is comparatively less abundant and more anatomically restricted than that of D1 and D2 receptors. However, this does not detract from the overall importance of both D1-like and D2-like receptors in many cognitive, neuroendocrine and behavioural/motor functions.

A growing number of studies have implicated involvement of central D2-like receptors, in particular in psychiatric diseases such as schizophrenia (Dean et al, 2004; Grace, 1993; Vernaleken et al, 2004) and Parkinson's disease (Antonelli et al, 2006; Jenner, 2003; Robertson, 1992). In addition to mediating various cognitive and motor functions, there is now convincing pharmacological evidence for a role of the D2-like receptors in control of male sexual behaviour, particularly penile erection and ejaculation (Docherty et al, 1991; Clements et al, 2007; Melis et al, 2005). We have shown (in

Chapter 3) that during pharmacologically-induced penile erection, D2 and D3 but not D4 receptors may have some role in mediating penile erection. Furthermore, during physiologically-induced penile erection, D4 receptors appear to have a more prominent role than D2 and D3 receptors. So the different contexts in which penile erection is observed have shown there to be some convincing support for D2-like receptor involvement. Thus, establishing the precise cellular location and distribution of the D2-like receptor subtypes will help our understanding of dopamine receptor interactions and the importance of their role in emotionality and neuroendocrine-mediated behaviours.

4.1.1 D2 receptors

As explained in Chapter 1 (section 1.8.2) D2 receptors are widely and abundantly expressed throughout the rat brain. Cellular location of D2 receptors include the cell bodies (Brok et al, 1992) and dendritic spines (Levey et al, 1993) and, subcellularly they can be found near the golgi apparatus and at the plasma membrane (Takeuchi and Fukunaga, 2003). Various neuronal phenotypes are known to express D2 receptors such as GABA, enkephalin and substance P, (Moine and Bloch, 1996; Moine and Gaspar, 1998). Additionally, D2 receptor mRNA has been located on cholinergic and somatostatinergic neurons (MacLennan et al, 1994). However, oxytocin neuron expression of D2 receptors has not yet been established.

4.1.2 D3 receptors

D3 receptor expression is generally restricted to the limbic regions (Levant, 1997 for review) (see Chapter 1, section 1.8.2). Unlike the D2 and D4 receptors, subcellular location and distribution of D3 receptors within the cells remains unknown. Although substance P-, enkephalin- and neurotensin containing neurons express D3 receptors (Diaz et al, 1995; Moine and Bloch, 1996), the presence of D3 receptors on oxytocin neurons remains unknown.

4.1.3 D4 receptors

D4 receptors are preferentially expressed in cortical and some subcortical regions (see Chapter 1, section 1.8.2). At the ultrastructural level D4 receptors are often associated with the plasma membrane, endoplasmic reticulum and the cytoplasm (Mrzljak et al, 1996). Additionally, D4 receptors are highly expressed in GABAergic neurons in the cortex, hippocampus and substania nigra (Mrzljak et al, 1996; Wedzony et al, 2000) and D4 receptor mRNA is localised on a small population of Substance P neurons in the neostriatum (Surmeier et al, 1996). However, as with D2 and D3 receptors, the presence of D4 receptors on oxytocin cells has yet to be reported.

4.1.4 D2, D3 and D4 receptor expression in the MPN, SON and PVN

The hypothalamus has been shown to possess the densest population of D2-like receptors in comparison to other brain areas in the rat (Khan et al, 1998). Also, as previously stated, hypothalamic nuclei, namely the MPN and PVN, are particularly responsive to the pro-erectile effects of D2-like dopaminergic ligands (Eaton et al, 1991; Melis et al, 2005; Moreland et al, 2005; Moses et al, 1995). Because these three nuclei contain moderate to large oxytocinergic populations, which have also been implicated in male sexual function (Kita et al, 2006; Pattig et al, 2005; Rampin, 2004 for review), it is of immense interest to investigate whether oxytocin neurons in the MPN, SON and PVN express D2-like receptors. It certainly seems likely that D2-like receptors are located on oxytocin cells due to the large number of pharmacological studies implicating central oxytocin pathways in the mediation of dopaminergic actions (Cameron et al, 1992; Melis et al, 1990; Uvnas-Moberg et al, 1995). Thus, it seems that oxytocinergic expression of D2-like receptors in the MPN, SON and PVN would provide direct anatomical evidence for a dopamine-oxytocin link within these nuclei and potentially indicate dopaminergic control of hypothalamic oxytocin neurons.

MPN

D2 and D3 receptor mRNA are moderately expressed in the MPN (Bouthenet et al, 1991; Gurevich and Joyce, 1999). In addition, binding studies have shown there to be a particularly high density of dopamine D4 receptor binding sites in the rat MPN (Primus et al, 1997).

SON

In the SON, D2 and D4 receptors are moderately expressed on magnocellular neurons (Defagot et al, 1997; Maltais et al, 2000). However, to our knowledge, D3 receptor expression in the SON has not yet been established.

PVN

The PVN expresses moderately dense populations of both D2 and D3 receptor mRNA (Bouthenet et al, 1991). Additionally, high levels of D2 receptor protein are also found in this nucleus. There is a high degree of D4 receptor immunolabelling of the magnocellular PVN, however, D4 receptor immunolabelling in the parvocellular PVN is comparatively lower (Bitner et al, 2006; Defagot et al, 1997).

While it is apparent that the D2-like receptors are present in relatively dense populations in the MPN, SON and PVN, the neuronal phenotypes expressing these receptors remain unknown. In the PVN and SON, the oxytocinergic cell groups comprise approximately 40-50% of the total neuronal population; therefore, it is tempting to suggest that a proportion of these hypothalamic oxytocin cells must express D2, D3 and/or D4 receptors.

Thus, our hypothesis was that D2, D3 and D4 receptors are expressed by oxytocin neurons in the MPN, SON and PVN of sexually-experienced male rats. In order to demonstrate this, double immunofluorescence for either the D2, D3 or D4 receptor and oxytocin was performed on perfused-fixed brain sections containing the MPN, SON and PVN. To further investigate the approximate total

percentage of oxytocin cells expressing D2-like receptors in each nuclei, a preliminary quantitative measure was applied.

Chapter 4

4.2 Methods

Dopamine D2, D3 and D4 receptor expression in the MPN, SON and PVN

4.2.1 Animals

Male Sprague-Dawley rats (200-250g) were used and housed the normal light cycle on a 12:12h light dark cycle (lights on 7:00am to 7:00pm). Sexually-experienced male rats were used to remain consistent with those animals used in the sexual behaviour experiment. Sexual experience was gained by placing a male with a receptive female over 2-3 nights, copulation through to ejaculation was confirmed by the presence of a vaginal plug of semen. This was repeated twice to allow 2 runs of sexual experience. Those males who did not mate after 4 days of being placed with a female were excluded from the study. Approximately, one week later rats were perfused fixed and brains stored as explained in the General Methods.

4.2.2 Immunofluorescence

Antibodies

Anti-D2, -D3 and –D4 receptor polyclonal antibodies (rabbit anti-rat, all Calbiochem, UK) have been used in a previous study demonstrating dopamine receptor expression in the choroid plexus (Mignini et al, 2000). After establishing an antibody dilution curve for each dopamine receptor expression in the rat brain, it was decided to use the D2, D3 and D4 receptor antibody at a 1:1000, 1:3000 and 1:1000 dilutions, respectively. We used an anti-oxytocin monoclonal antibody (mouse anti-rat, Chemicon, UK), which has been used previously (Brailiou et al, 2007) at a dilution of 1:5000 so this dilution was subsequently used. The secondary fluorescent antibodies, namely, Alexa-fluor 488 (goat anti-rabbit) and Alexa-fluor 568 (goat anti-mouse) (both $(Fab')_2$ fragments from Invitrogen, UK) have been applied and published in previous studies (Granneman et al, 2007; Croze et al, 2000; Matsumoto et al, 2005) at approximate dilutions of 1:1000 which we used for all experiments that followed.

4.2.3 Cell quantification

A simplisitic and preliminary quantification method was used to establish the percentage of oxytocin cells in the MPN, SON and PVN expressing D2, D3 or D4 receptors. Using 3D images created by the confocal microscope and the associated Zeiss computer software, we were able track dopamine receptor and oxytocin expression throughout the entire tissue (three sections per area per rat, n=3) due to the combined sequential scanning of thinner specimen slices. The number of cells positive for the D2, D3, D4 receptor or oxytocin and co-localised dopamine receptor and oxytocin were counted separately. The incidence of co-localisation was expressed as the percentage of dopamine receptor and oxytocin cells / the total number of oxytocin cells. Values were calculated per region per rat and then per group. Statistics could not be performed due to the low "n" number. However, such basic semi-quantitative anlaysis should give an indication of the proportion of oxytocin cells expressing dopamine receptors and thus shed some light on potential dopaminergic and oxytocinergic interactions in hypothalamic nuclei.

Chapter 4

4.3 Results

4.3.1 D2, D3 or D4 receptor and oxytocin expression in the MPN

D2 receptor

There was abundant D2 receptor expression in the MPN. D2 receptor labelling was primarily restricted to the cytoplasm and cell membrane of oxytocin cells but did not appear to be expressed on oxytocinergic fibres. Interestingly, there was also some intense staining of D2 receptors on non-oxytocinergic cell bodies and cellular processes in the MPN (Figure 4.1).

D3 receptor

There were moderately high levels of D3 receptor expression in the MPN. Cellular distribution of the D3 receptor was quite diverse with the cytoplasm, cell bodies and almost all dendritic processes of oxytocin neurons expressing the D3 receptor in the MPN. Additionally, several non-oxytocinergic neurons were seen to express D3 receptors (Figure 4.2).

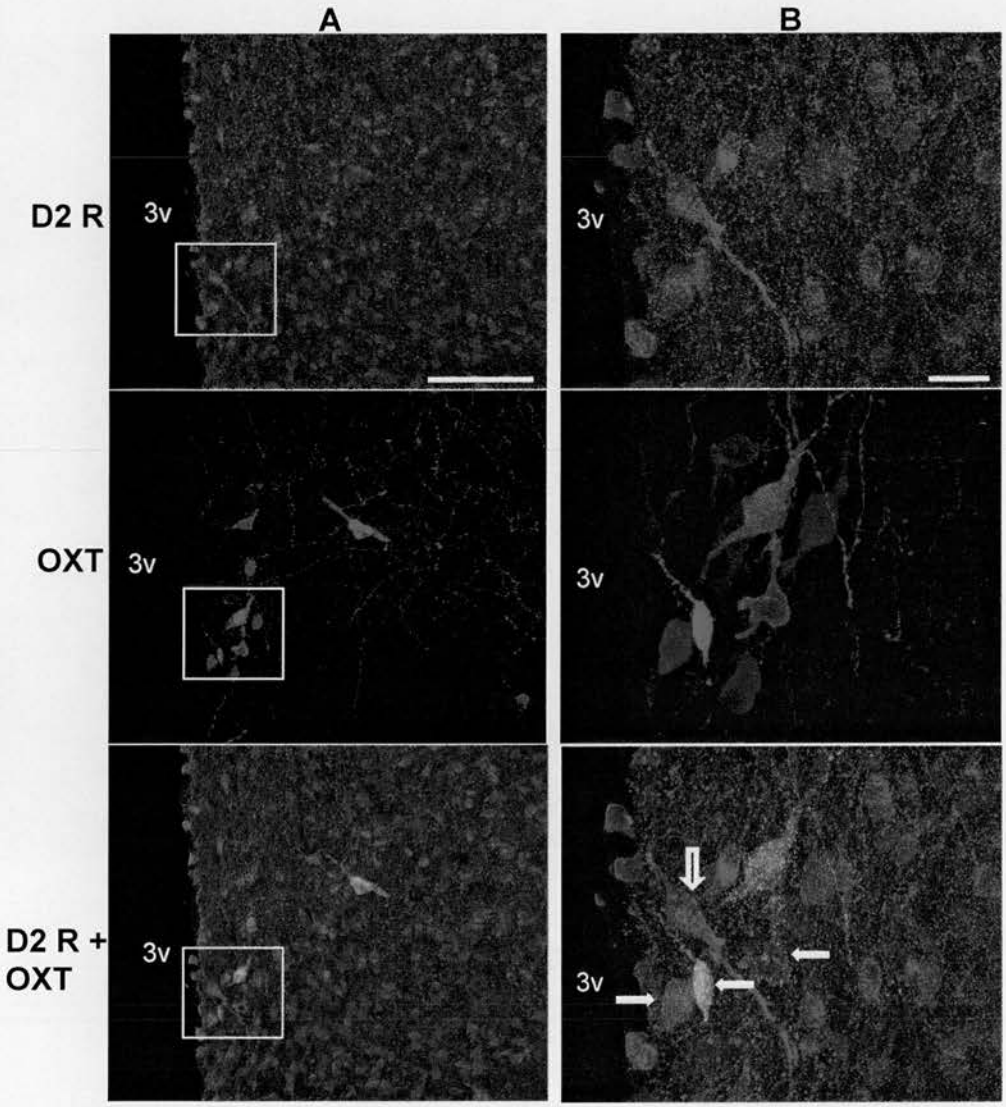
D4 receptor

The D4 receptor signal was comparatively weaker in the MPN when compared to the D2 and D3 receptors, however, it did appear to be expressed in the cytoplasm, on the cell body and dendritic process of the oxytocin cell shown in the photomicrographs (Figure 4.3). As with the D2 and D3 receptor, D4 receptors were also expressed on cell bodies and processes of cells that were not oxytocinergic.

Percentage co-localisation in the MPN

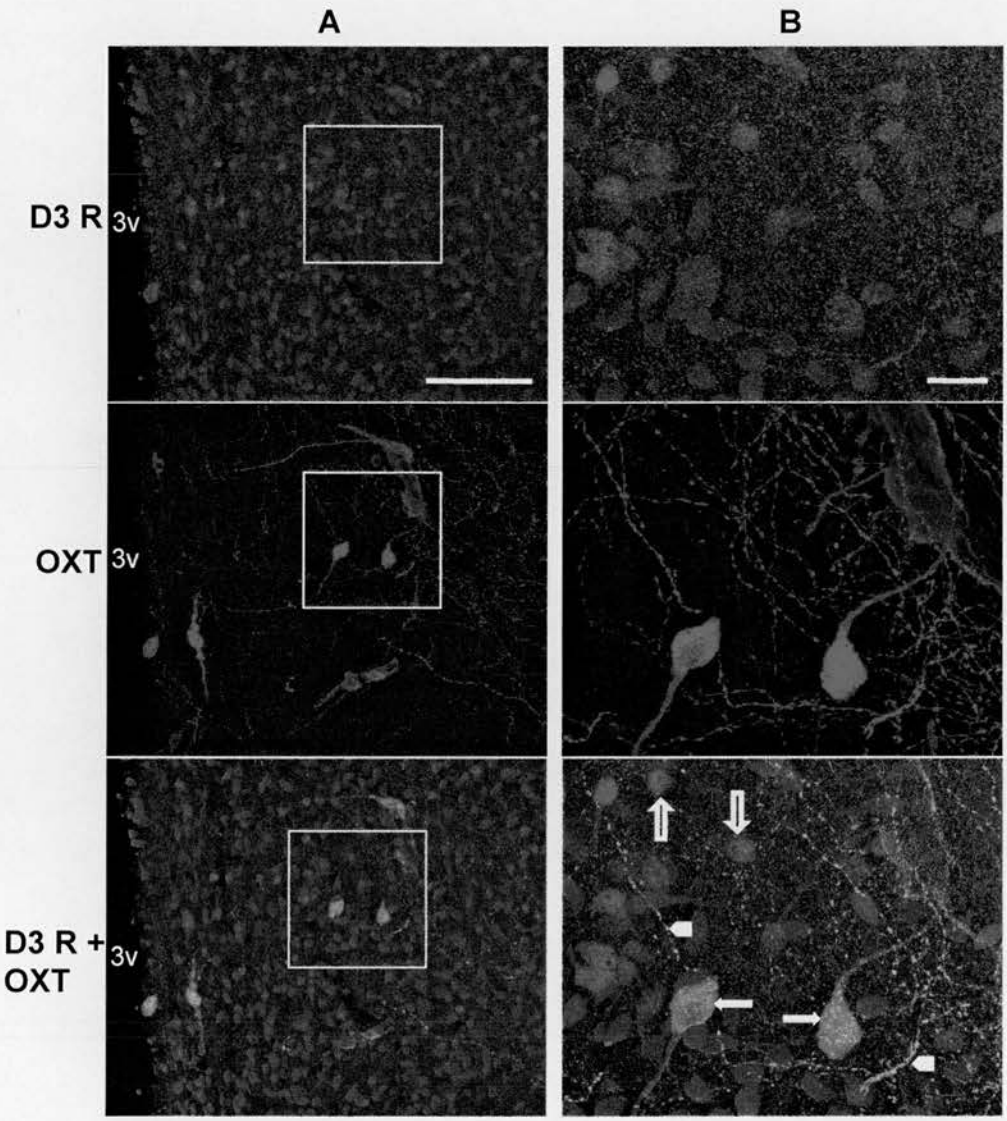
Figure 4.10A illustrates the percentage of oxytocin neurons expressing either the D2, D3 or D4 receptor in the MPN of sexually-experienced male rats. It could be seen that MPN oxytocin cells

Figure 4.1 : D2 receptor and oxytocin expression in the MPN



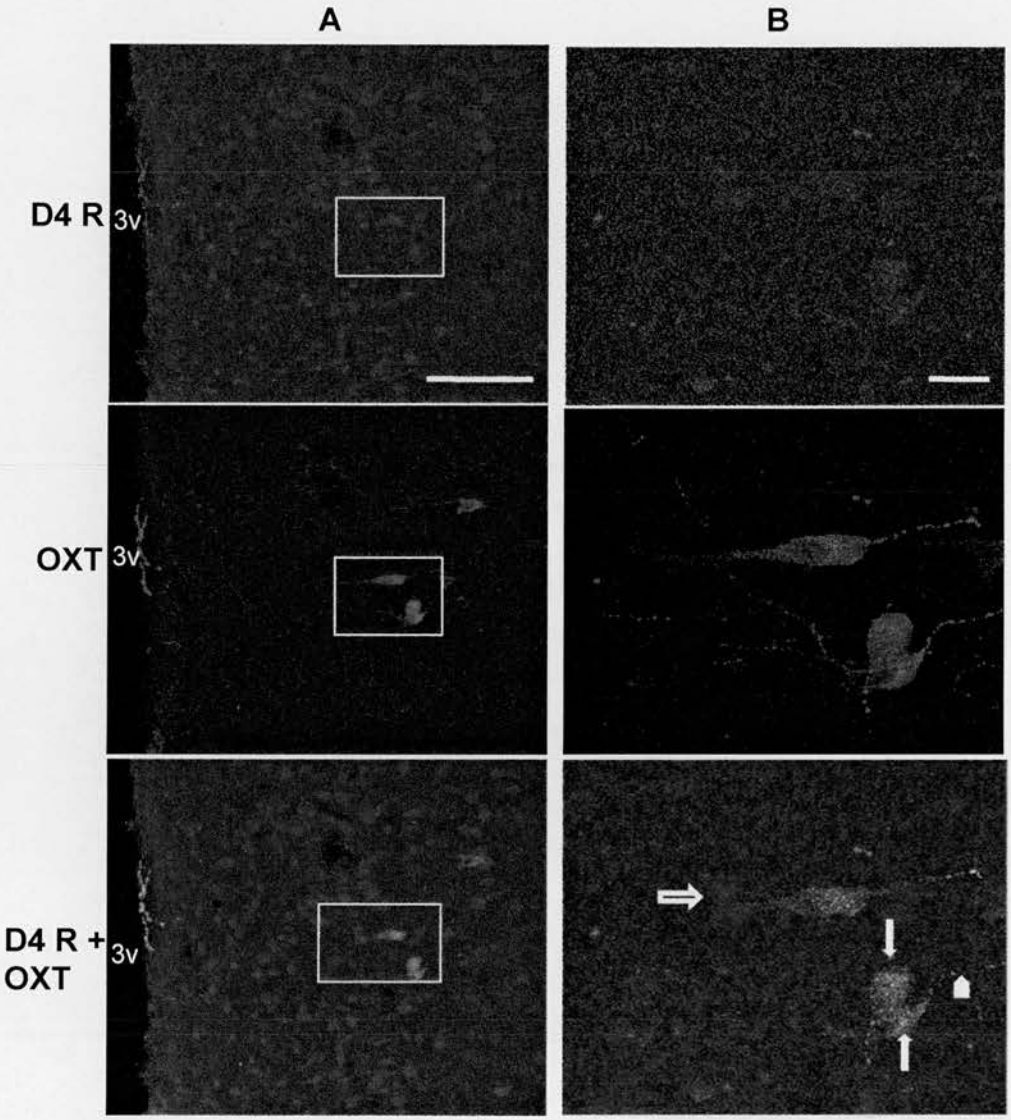
The co-expression of the D2 receptor and oxytocin in the MPN. D2 receptor (D2R) and oxytocin (OXT) labelling are shown in green and red respectively. An overlap of green and red labelling (yellow in colour) indicates a D2-expressing oxytocin cell. Co-localisation of the D2 receptor and oxytocin are shown in column A and the areas highlighted in A are shown at higher magnification in column B. D2 receptors were found to be expressed on the cell bodies of oxytocin neurons in the MPN. Filled arrows indicate a co-localised cell and empty arrows show D2 receptor or oxytocin labelling only. Scale bars represent 100μm and 20μm in A and B respectively. 3v=third ventricle

Figure 4.2 : D3 receptor and oxytocin expression in the MPN



The co-expression of the D3 receptor (D3R) and oxytocin (OXT) in the MPN. D3 receptor and oxytocin labelling are shown in green and red respectively. An overlap of green and red labelling (yellow in colour) indicates a D3-expressing oxytocin cell. Co-localisation of the D3 receptor and oxytocin are shown in column A and the areas highlighted in A are shown at higher magnification in column B. D3 receptors were found to be expressed on the cell bodies of oxytocin neurons in the MPN. Filled arrows indicate co-localised cell bodies, arrowheads illustrate co-localised fibres and empty arrows show D3 receptor or oxytocin labelling only. Scale bars represent 100μm and 20μm in A and B respectively. 3v=third ventricle.

Figure 4.3 : D4 receptor and oxytocin expression in the MPN



The co-expression of the D4 receptor (D4R) and oxytocin (OXT) in the MPN. D4 receptor and oxytocin labelling are shown in green and red respectively. An overlap of green and red labelling (yellow in colour) indicates a D4-expressing oxytocin cell. Co-localisation of the D4 receptor and oxytocin are shown in column A and the areas highlighted in A are shown at higher magnification in column B. D4 receptors were found to be expressed on the cell bodies and cellular processes of oxytocin neurons in the MPN. Filled arrows indicate co-localised cell bodies, arrowheads illustrate co-localised fibres and empty arrows show D4 receptor or oxytocin labelling only. Scale bars represent 100μm and 20μm in A and B respectively. 3v=third ventricle.

abundantly expressed the dopamine D3 receptor (68%), whilst the D2 receptor was moderately expressed (40%) and there was comparatively lower levels of the D4 receptor labelling (15%).

4.3.2 D2, D3 or D4 receptor and oxytocin expression in the SON

D2 receptor

In the SON, punctuate labelling of the D2 receptor was widely expressed. Oxytocin neurons seemed to express the D2 receptor on the cell bodies but not on the dendritic processes. As seen in the MPN, not all oxytocin neurons were co-localised with D2 receptors as evidenced by D2 receptor expression on non-oxytocinerigc cells in the SON (Figure 4.4).

D3 receptor

The D3 receptors were expressed in the SON. Oxytocin neurons seemed to express the D3 receptor on the cell bodies but not on the dendritic processes; however, D3 receptor staining was less punctuate than the D2 receptor labelling in the SON. As seen in the MPN, D3 receptors were expressed on non-oxytocinergic cells in the SON (Figure 4.5).

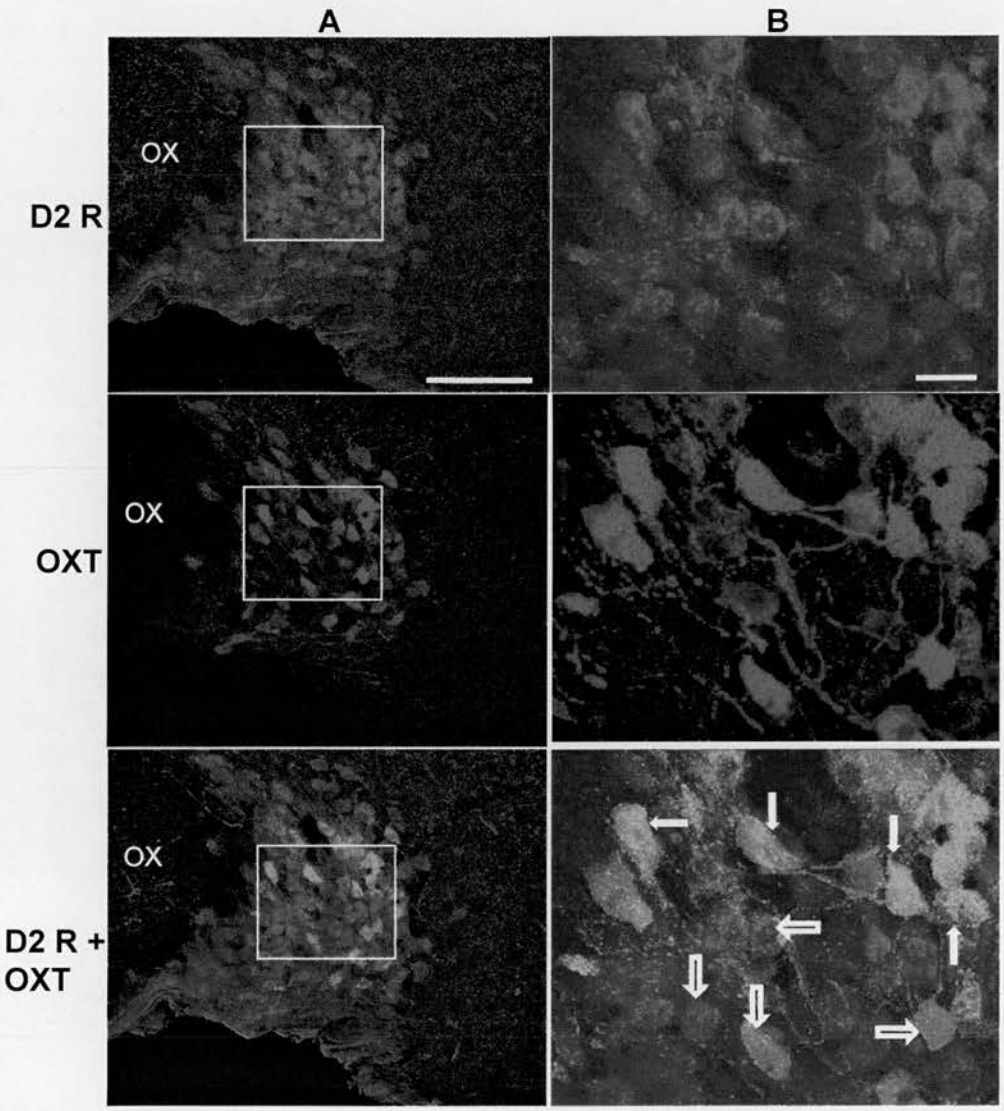
D4 receptor

The D4 receptor was widely expressed in the SON. Oxytocin neurons seemed to express the D4 receptor in the cytoplasm and on the cell bodies but not on the dendritic processes. As seen in the MPN, D4 receptors were expressed by cells that were not oxytocinerigc (Figure 4.6).

Percentage co-localisation in the SON

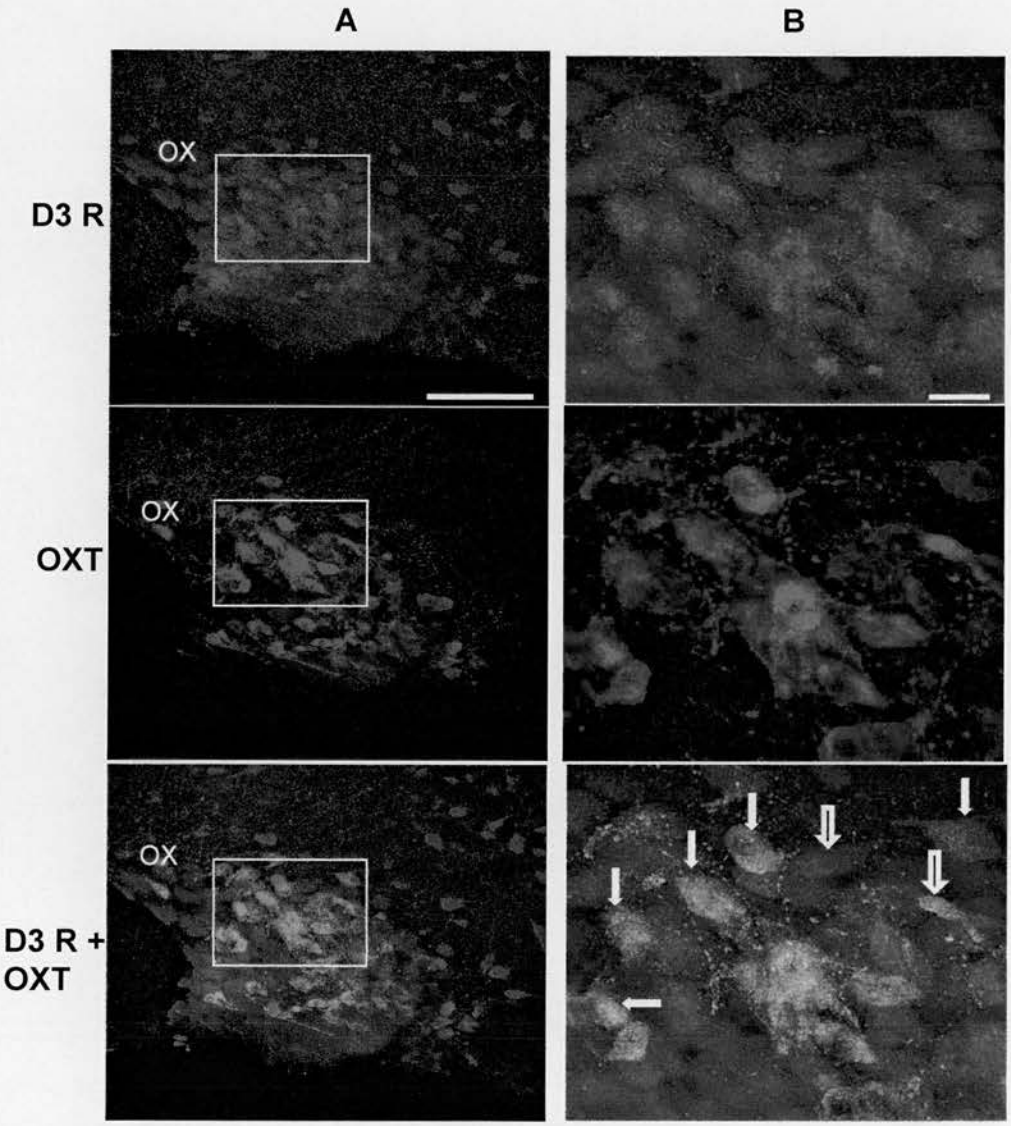
Figure 4.10B illustrates the percentage of oxytocin neurons expressing either the D2, D3 or D4 receptor in the SON of sexually-experienced male rats. SON oxytocin cells expressed low to moderate levels of dopamine D3 receptors (26%), whilst the D2 and D4 receptors were expressed in relatively low levels on oxytocin neurons (14% and 15% respectively).

Figure 4.4 : D2 receptor and oxytocin expression in the SON



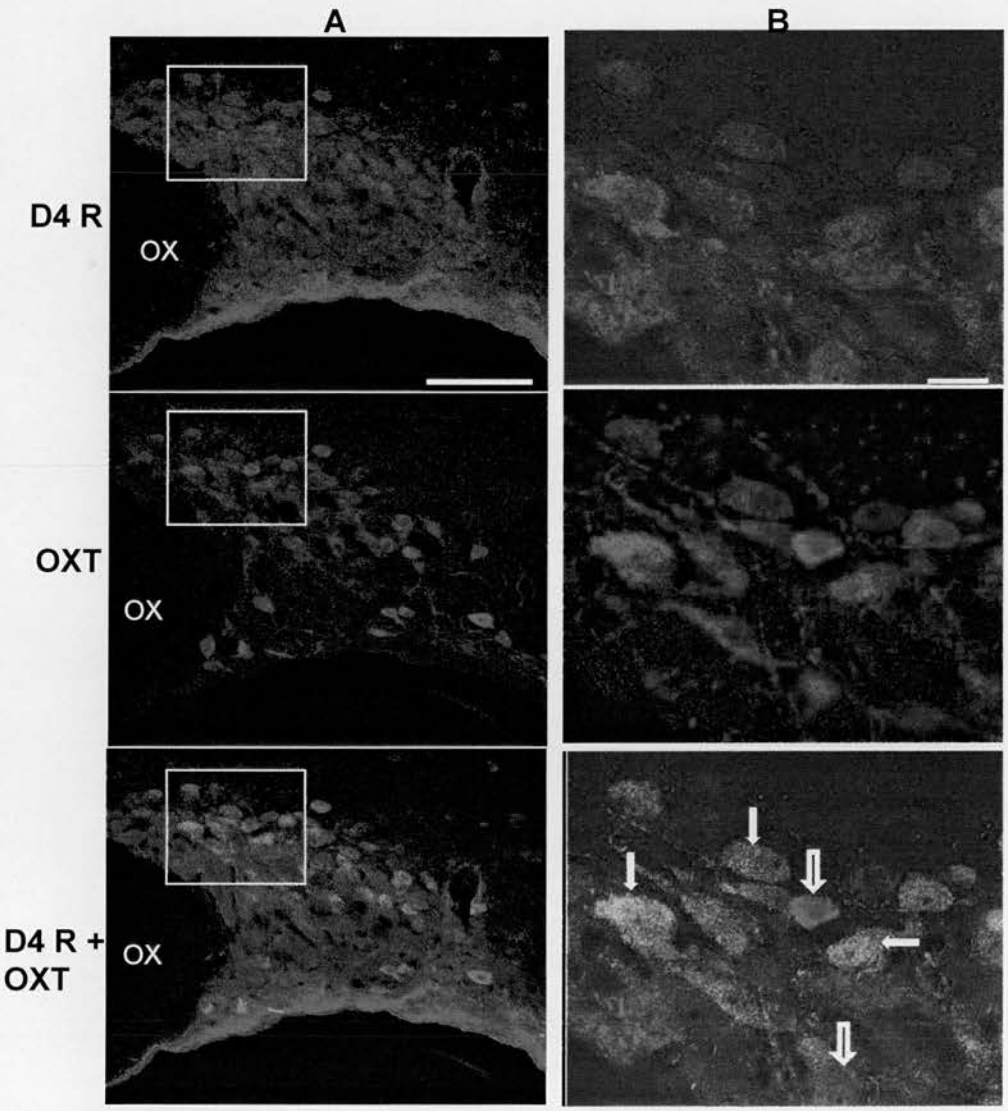
The co-expression of the D2 receptor (D2R) and oxytocin (OXT) in the SON. D2 receptor and oxytocin labelling are shown in green and red respectively. An overlap of green and red labelling (yellow in colour) indicates a D2-expressing oxytocin cell. Co-localisation of the D2 receptor and oxytocin are shown in column A and the areas highlighted in A are shown at higher magnification in column B. D2 receptors were found to be expressed on the cell bodies but not the cellular processes of oxytocin neurons in the SON. Filled arrows indicate co-localised cell bodies and empty arrows show D4 receptor or oxytocin labelling only. Scale bars represent 100μm and 20μm in A and B respectively. OX=optic chiasm.

Figure 4.5 : D3 receptor and oxytocin expression in the SON



The co-expression of the D3 receptor (D3R) and oxytocin (OXT) in the SON. D3 receptor and oxytocin labelling are shown in green and red respectively. An overlap of green and red labelling (yellow in colour) indicates a D3-expressing oxytocin cell. Co-localisation of the D3 receptor and oxytocin are shown in column A and the areas highlighted in A are shown at higher magnification in column B. D3 receptors were found to be expressed in the cytoplasm and on the cell bodies but not the cellular processes of oxytocin neurons in the SON. Filled arrows indicate co-localised cell bodies and empty arrows show D3 receptor or oxytocin labelling only. Scale bars represent 100μm and 20μm in A and B respectively. OX=optic chiasm.

Figure 4.6 : D4 receptor and oxytocin expression in the SON



The co-expression of the D4 receptor (D4R) and oxytocin (OXT) in the SON. D4 receptor and oxytocin labelling are shown in green and red respectively. An overlap of green and red labelling (yellow in colour) indicates a D4-expressing oxytocin cell. Co-localisation of the D4 receptor and oxytocin are shown in column A and the areas highlighted in A are shown at higher magnification in column B. D4 receptors were found to be expressed on the cell bodies but not the cellular processes of oxytocin neurons in the SON. Filled arrows indicate co-localised cell bodies and empty arrows show D4 receptor or oxytocin labelling only. Scale bars represent 100μm and 20μm in A and B respectively. OX=optic chiasm.

D2, D3 or D4 receptor and oxytocin expression in the PVN

D2 receptor

D2 receptors were expressed by a small population of oxytocin neurons in the PVN. Co-expression was found mainly on magnocellular neurons and to a lesser extent on parvocellular oxytocin neurons. Oxytocin neurons seemed to express the D2 receptor in the cytoplasm and on the cell bodies but not on the dendritic processes. As seen in the MPN, D2 receptors were also expressed on non-oxytocinergic cells in the PVN (Figure 4.7).

D3 receptor

D3 receptors were expressed moderately by oxytocin neurons in the PVN. Co-expression was found on magnocellular and parvocellular oxytocin neurons in the PVN. Oxytocin neurons seemed to express the D3 receptor on their cell bodies and dendritic fibres. As seen in the MPN, D3 receptors were also expressed on non-oxytocinergic cells in the PVN (Figure 4.8).

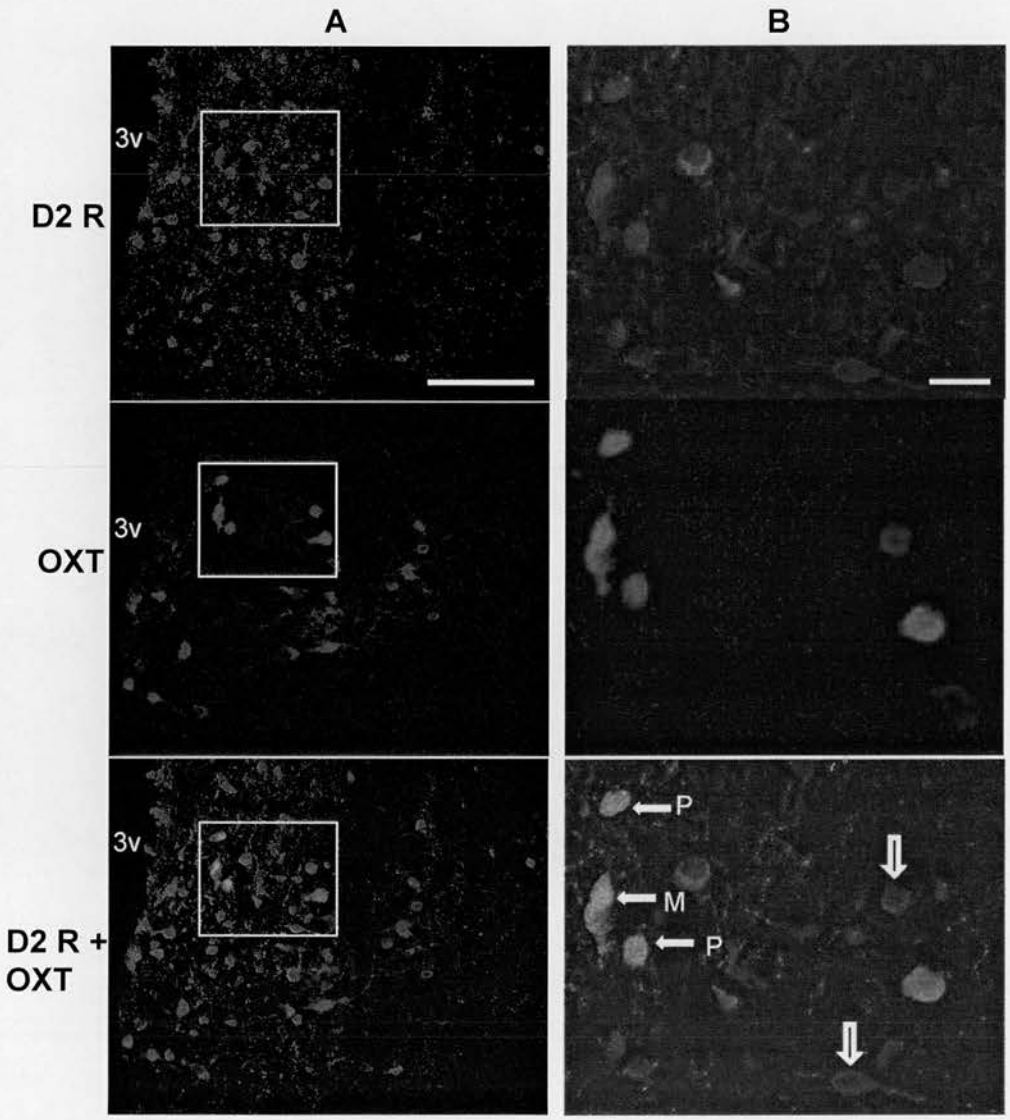
D4 receptor

D4 receptors were expressed moderately by oxytocin neurons in the PVN. There was clear punctuate labelling on the oxytocin cell bodies. Co-expression was found exclusively on magnocellular oxytocin neurons in the PVN. In the sections analysed, parvocellular oxytocin cells did not appear to express D4 receptors. As seen in the MPN, D4 receptors were also expressed on non-oxytocinerigc cells in the PVN (Figure 4.9).

Percentage co-localisation in the PVN

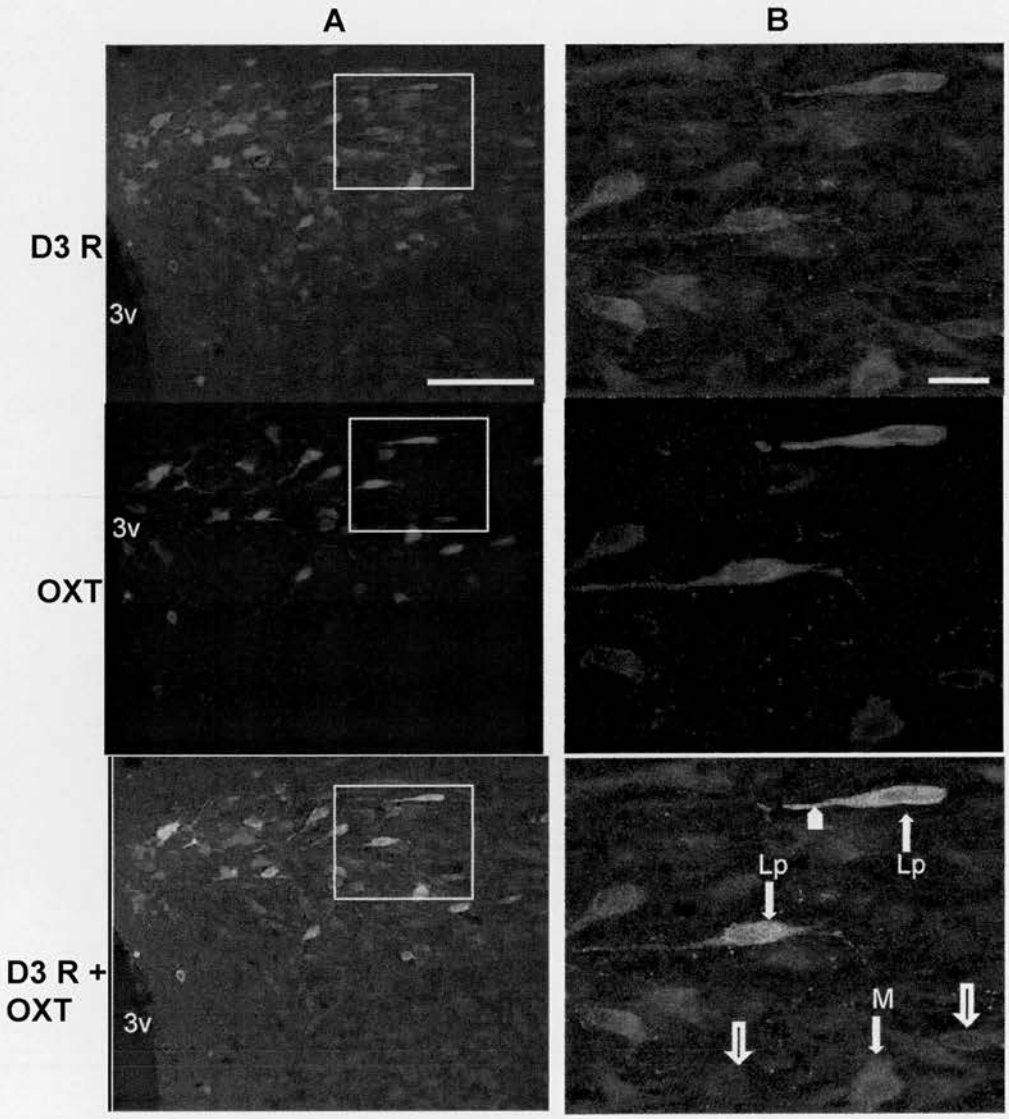
Figure 4.10C illustrates the percentage of oxytocin neurons expressing either the D2, D3 or D4 receptor in the PVN of sexually-experienced male rats. It could be seen that PVN oxytocin cells expressed consistently low to moderate levels of dopamine D2, D3 and D4 receptors (21%, 21% and 30% respectively).

Figure 4.7 : D2 receptor and oxytocin expression in the PVN



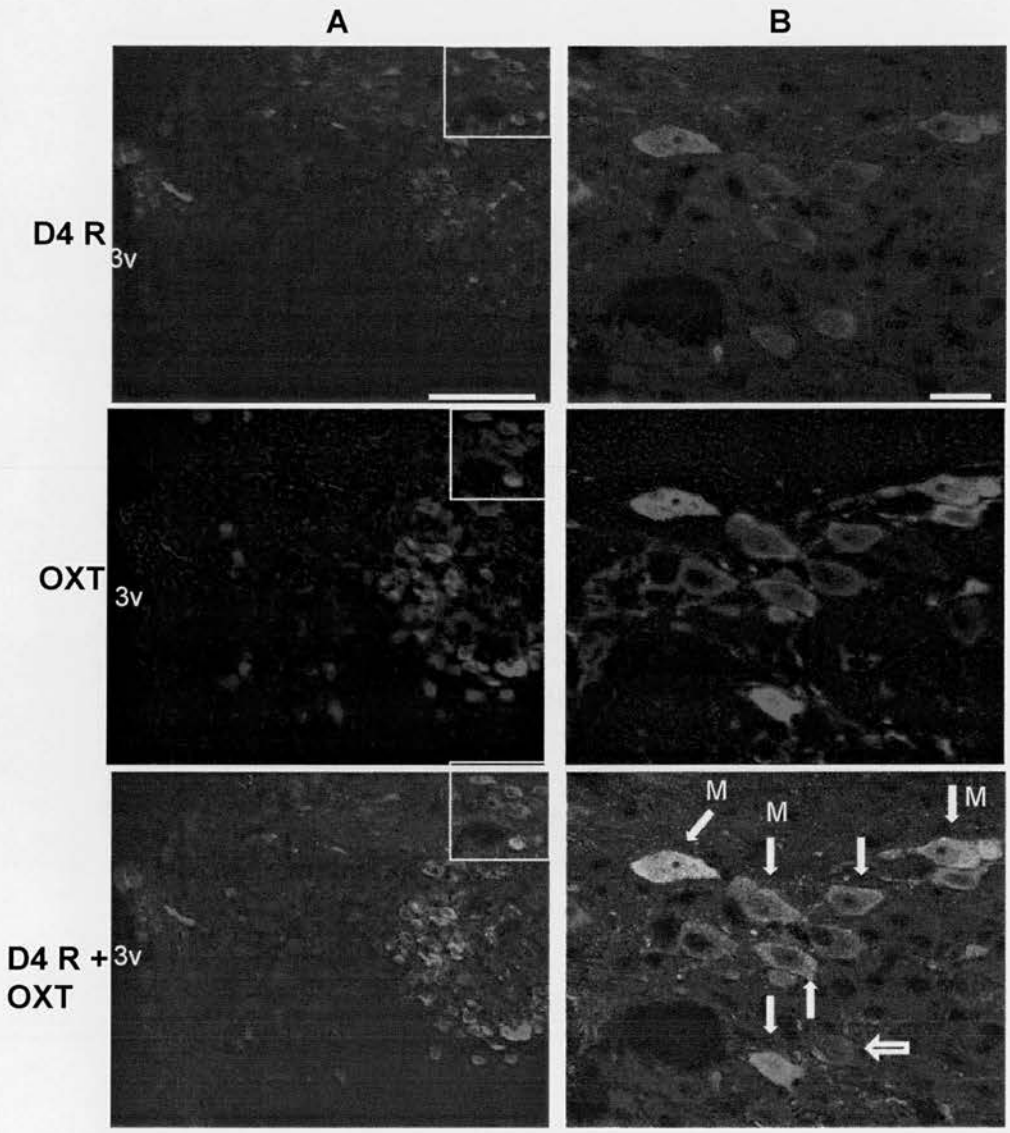
The co-expression of the D2 receptor (D2R) and oxytocin (OXT) in the PVN. D2 receptor and oxytocin labelling are shown in green and red respectively. An overlap of green and red labelling (yellow in colour) indicates a D2-expressing oxytocin cell. Co-localisation of the D2 receptor and oxytocin are shown in column A and the areas highlighted in A are shown at higher magnification in column B. D2 receptors were found to be expressed on the cell bodies of oxytocin neurons in the PVN. Filled arrows indicate co-localisation and empty arrows show D2 receptor or oxytocin labelling only. Scale bars represent 100μm and 20μm in A and B respectively. 3v=third venricle; P=parvocellular oxytocin cell; M=magnocellular oxytocin cell.

Figure 4.8 : D3 receptor and oxytocin expression in the PVN



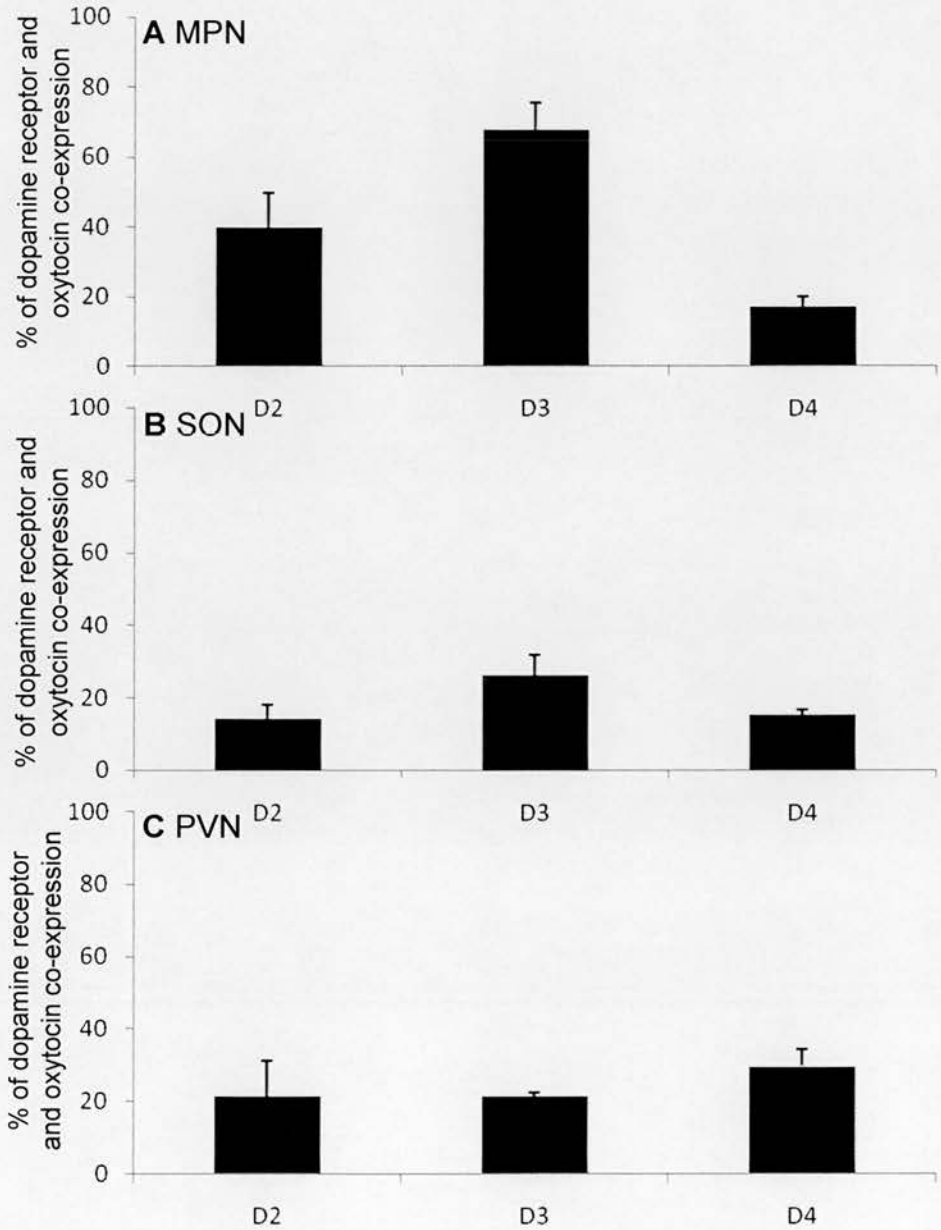
The co-expression of the D3 receptor (D3R) and oxytocin (OXT) in the PVN. D3 receptor and oxytocin labelling are shown in green and red respectively. An overlap of green and red labelling (yellow in colour) indicates a D3-expressing oxytocin cell. Co-localisation of the D3 receptor and oxytocin are shown in column A and the areas highlighted in A are shown at higher magnification in column B. D3 receptors were found to be expressed on the cell bodies and dendritic fibres of oxytocin neurons in the PVN. Filled arrows indicate co-localised cell bodies, arrowheads illustrate co-localised dendritic fibres and empty arrows show D3 receptor or oxytocin labelling only. Scale bars represent 100μm and 20μm in A and B respectively. 3v=third ventricle; Lp-lateral parvocellular oxytocin cell; M=magnocellular oxytocin cell.

Figure 4.9 : D4 receptor and oxytocin expression in the PVN



The co-expression of the D4 receptor (D4R) and oxytocin (OXT) in the PVN. D4 receptor and oxytocin labelling are shown in green and red respectively. An overlap of green and red labelling (yellow in colour) indicates a D4-expressing oxytocin cell. Co-localisation of the D4 receptor and oxytocin are shown in column A and the areas highlighted in A are shown at higher magnification in column B. D4 receptors were found to be expressed on the cell bodies but not the dendritic fibres of oxytocin neurons in the PVN. Filled arrows indicate co-localised cell bodies and empty arrows show D4 receptor or oxytocin staining only. Scale bars represent 100μm and 20μm in A and B respectively. 3v=third venricle; M=magnocellular oxytocin cell.

Figure 4.10 : Co-expression of dopamine receptors and oxytocin in the MPN, SON and PVN



Percentage of oxytocin neurons in the MPN (A), SON (B) and PVN (C) expressing either the D2, D3 or D4 receptor. Similar levels of co-localisation of oxytocin and D2, D3 or D4 receptor were observed in the SON and PVN. Comparatively more D3 receptor and oxytocin co-localisation was seen in the MPN compared to D2 or D4 receptor co-localisation with oxytocin.

Chapter 4

4.4 Discussion

Behavioural studies that we have conducted showing the effects of dopamine agonists and antagonists on oxytocin neuron activity in the MPN, SON and PVN, provide support for a direct dopaminergic input to hypothalamic oxytocin neurons. The present study of dopamine D2-like receptor immunofluorescent labelling in the rat hypothalamus showed distinct cellular co-localisation of D2, D3 and D4 receptors on oxytocin neurons in the MPN, SON and PVN of sexually-experienced male rats. There were some subtle differences in regional and cellular distribution of D2-like receptors on oxytocin neurons in these three brain nuclei suggesting that D2, D3 and/or D4 receptors may differentially modulate hypothalamic oxytocin cells dependent on their anatomical and cell-specific location on oxytocin neurons.

4.4.1 Specificity of antibodies

Brain regions known to express abundant populations of the D2, D3 and D4 receptors were used as positive controls in this study (Figure 2.6, in General Methods). The caudate putamen (for the D2 and D4 receptor) and the olfactory tubercle (for the D3 receptor) were analysed after each immunofluorescence run, to confirm that there was D2-like receptor region-specific labelling. In those negative controls where the dopamine D2-like receptor antibody was omitted from the incubation medium, no receptor labelling was observed in any area of the brain including those areas that served as positive controls. In another group of negative controls, the dopamine D2-like receptor antibody was substituted with immune serum from which the primary antibody was raised in (in this case normal rabbit serum). Brain sections immersed in this incubation medium showed relatively low levels of immuno-labelling. Thus, there is a possibility that these D2-like receptor antibodies display low levels of cross-reactivity to other unrelated proteins.

4.4.2 D2-like receptor expression on oxytocin neurons in the MPN, SON and PVN

MPN

The MPN is one of the most crucial brain areas involved in dopamine-mediated copulatory behaviour (Paredes and Agmo, 2004 for review). It is also highly sensitive to the pro-copulatory effects of apomorphine (Hull et al, 1986). Thus, it seems likely that D2, D3 and D4 receptors would be abundantly expressed in this nucleus. However, that the MPN expresses these receptors and on what cell type has not been established. For the first time, we show that D2, D3 and D4 receptors are expressed in the MPN. Additionally, a discrete population of oxytocin neurons within this nucleus express D2, D3 or D4 receptors. At high magnification D2, D3 and D4 receptors were found to be expressed on the cell bodies and on the cell membrane perimeter of MPN oxytocin neurons. Interestingly, D3 and D4 but not D2 receptors were located on oxytocinergic dendritic processes which suggests that D3 and D4 receptors in the MPN may mediate dendritic release of oxytocin postsynaptically. Additionally, in the MPN, high levels of oxytocin and D3 receptor co-localisation were observed. Thus, there seems to be a strong neuroanatomical basis for D3-receptor mediated activation of oxytocin neurons in the MPN.

SON

Only recently has the SON been implicated in male sexual function (Caquineau et al, 2006; Pattij et al, 2005). However, the responsiveness of the SON to the previously described pro-erectile dopaminergic ligands remains unkown. Thus, it was important to investigate potential cellular distribution of D2-like receptors on oxytocin neurons within this nucleus. Dopamine increases the firing rate of supraoptic oxytocin neurons *in vitro* (Mason, 1983). Moreover, D2 receptor agonists have been shown to depolarise supraoptic oxytocin neurons (Yang et al, 1991) and D4 receptors are known to be expressed in the SON (Defagot et al, 1997). Thus, from such studies it was speculated that D2 and perhaps D4 receptors were located on magnocellular oxytocin neurons. However, to our knowledge no such evidence was available for the D3 receptor.

Supraoptic oxytocin neurons were found to express D2, D3 and D4 receptors in similarly moderate levels. However, such D2-like receptor distribution appeared to be anatomically restricted to the cell

bodies of oxytocin neurons. D2-like immunolabelling of the dendritic fibres in the SON was not observed in the limited number of sections analysed. It has been suggested that dopaminergic ligands can alter oxytocinergic transmission in the SON indirectly via modulating GABAergic and/or glutamatergic transmission (Azdad et al, 2001; Price and Pittman, 2001). However, here we show that dopamine could excite oxytocin neurons via a more direct mechanism by acting on D2, D3 and / or D4 receptors located in the cell membrane of supraoptic oxytocin cells.

PVN

As with the MPN, the PVN is a moderately important nucleus in the control of male sexual behaviour (Paredes and Agmo, 2004 for review) and it is also highly responsive to dopaminergic agonists (particularly D4 agonists) (Docherty and Wisler, 1991; Melis et al, 2005, Paredes and Agmo, 2004). Pharmacological studies have shown that paraventricular dopaminergic and oxytocinergic pathways interact to facilitate penile erection (Argiolas and Melis, 2005, for review). However, the intricate signalling pathways subserving dopamine-oxytocin-mediated penile erection are still unclear. Dopaminergic fibres lie in close apposition to parvocellular and magnocellular neurons in the PVN (Decavel et al, 1987) thus it was logical to assume that paraventricular oxytocin neurons possessed D2-like receptors. This study revealed that D2, D3 and D4 receptors were abundantly expressed on the cell bodies of predominantly magnocellular and a few parvocellular oxytocin neurons in the PVN. However, only D3 receptors were found to be expressed in the dendritic processes of magnocellular oxytocin cells. Thus, it seems in the PVN dopamine may act directly on D2, D3 and D4 receptors located on oxytocin cell bodies. Additionally, dopamine may act via postsynaptic D3 receptors located on oxytocinergic dendrites.

4.4.3 Expression of D2-like receptors on non-oxytocinerigc neurons

Whilst hypothalamic oxytocin cells possess D2-like receptors, there appeared to be abundant expression of D2, D3 and D4 receptors on non-oxytocinergic neurons in the MPN, SON and PVN. These neurons may represent other cell groups implicated in regulating male sexual behaviour, such as serotonergic, dopaminergic, melancortinergic, vasopressinergic, galaninergic, GABAergic, glutamatergic and gonadotrophinergic phenotypes (Benelli et al, 1994; Caquineau et al, 2006;

Dennison et al 1996; Hull et al, 2004; Melis et al, 2006; 2001; Morris, 2003; Murphy et al, 1987; Ogier et al, 2006; Paredes and Agmo, 2004). In regard to the SON in particular, we know that this nucleus is comprised almost exclusively of oxytocin and vaspopressin cells (although oxytocin and vasopressin can be colocalised with other neuromediators). Since we have shown moderate levels of D2-like receptor expression on SON cells that are not oxytocin we can be almost certain that these are vasopressinergic. To our knowledge, there have been no reports as yet of vasopressin cells possessing dopamine D2-like receptors. This finding suggests that endogenous dopamine may also be able to modulate the activity of supraoptic vasopressin cells by acting directly on the soma of these cells. However, whether such a dopamine-vasopressin interaction underlies neural control of masculine sexual behaviour has yet to be established. Thus, it can be seen dopaminergic inputs to hypothalamic neurons are not restricted to oxytocin cells but in fact converge on a wide range of peptidergic neurons.

In this study, there were some technical limitations. In only three rats, three sections per brain region were analysed, thus it is difficult to suggest that these findings are representative of a group as a whole. Additionally, we were not able to examine subcellular location and distribution of the D2-like receptors. Applying electron microscopy would perhaps help in examining the intracellular presence of D2-like receptors and understanding how dopamine receptors regulate oxytocin release.

In conclusion, we have shown that D2, D3 and D4 receptors are expressed on hypothalamic oxytocin neurons. Our results provide a strong anatomical basis for a direct dopaminergic activation of oxytocin neurons in the MPN, SON and PVN. Moreover, D3 receptor expression on oxytocinergic dendritic processes in the MPN and PVN raises the possibility that dopamine may stimulate oxytocin release within each nucleus via D3 receptors. Although each receptor is expressed in the MPN, SON and PVN, it seems that D2 and D4 receptors are primarily expressed on oxytocin cell bodies, whilst D3 receptors are located somatodendritically. It is not clear if somatic and dendritic dopamine receptor activation differentially regulate oxytocin release. However, due to the apparent differences in cellular location, such a finding raises the possibility that D2-like dopamine receptors may in fact

stimulate hypothalamic oxytocin release via similar and/or alternate mechanistic pathways. The presence of D2-like receptors on the cell bodies and/or the dendritic processes of oxytocin neurons introduces the possibility of an additional dopaminergic control of somatodendritic oxytocin release. Perhaps dopamine and /or oxytocin itself operate in a positive feedback loop whereby dopamine- and/or oxytocin-induced oxytocin release (via direct activation of dopamine and/or oxytocin receptors on oxytocin cells), disinhibit GABAergic synaptic transmission and so increase the synchronous firing activity of oxytocin neurons as seen during lactation (de Kock et al, 2003), although this is highly speculative.

It is well known that oxytocin receptors are located on hypothalamic oxytocin neurons which facilitates oxytocin to stimulate its own release in a calcium-dependent manner (Lambert et al, 1994; Ludwig et al, 2002). During the female reproductive cycle, hypothalamic oxytocin cells become more excitable and their firing rate changes from low-frequency to high-frequency firing (Leng et al, 1999). Such an augmentation in the firing rate has been shown to be due to enhanced inhibition of GABAergic neurotransmission by increased somatodendritic release of oxytocin (de Kock et al, 2003). It seems entirely possible that such a somatodendritic regulatory mechanism of oxytocin release exists in the male rat during penile erection.

Oxytocinergic pathways in the hypothalamus do appear to be one particular population of target neurons that partly mediate the actions of dopamine during erectile function. Moreover, we show that oxytocin neurons within sexually responsive brain nuclei possess D2-like receptors, thus providing neuroanatomical evidence for a direct dopaminergic input to these oxytocin cells. If the activity of oxytocin cells is altered during dopamine ligand mediated- penile erection (as we have shown in chapter 3), then it seems logical to propose that such dopaminergic agents can influence oxytocin release within the CNS, providing they gain access to the brain. During sexual behaviour, there is increased oxytocin release within the PVN (Waldherr and Neumann, 2007). However, the lumbosacral spinal cord (containing pro-erectile centres) is another central candidate believed to mediate the effects of dopamine due to the presence of oxytocin receptor binding sites and innervating oxytocinergic fibres originating in the parvocellular PVN (Tang et al, 1998; Veronneau-Longueville et

al, 1999). Thus dopamine-mediated oxytocin release and action in the lumbosacral spinal cord of the male rat was next investigated.

Chapter 5

Lumbosacral oxytocin action and apomorphine-induced penile erection

Chapter 5

5.1 Introduction

Electrical and pharmacological stimulation of the PVN have been shown to induce robust increases in intracavenous pressure (ICP) in anaesthetised and conscious male rats (Bernabe et al, 1999; Chen et al, 1997). As previously explained (in General Methods, section 8.1), using ICP as a physiological marker of penile erection allows greater sensitivity and objectivity when quantitatively analysing erectile function. The corpora cavernosa erectile tissues lie alongside each side of the shaft of the penis and are larger in diameter in comparison to the corpora spongiosum (of which there is only one) which runs along the ventral surface of the penis. Penile pressure is generally recorded from the corpora cavernosum because (1) due to its size and shape, it is technically easier to insert a pressure probe into this region and (2) the majority of blood (90%) enters the corpora cavernosa during tumescence. Thus, recording from the corpus cavernosum rather than the corpus spongiosum will give a more accurate measurement of intra-penile pressure during the achievement of penile erection. The corpora spongiosum forms the glans penis and acts to prevent compression of the urethra during erection and facilitates movement of the ejaculate. There have been some studies measuring corpora spongiosum pressure during erection in rats, stallions, dogs and sheep (Beckett et al, 1975a; Beckett et al, 1975b; Purohit and Beckett, 1976; Schmidt et al, 1995). However, all these studies concomitantly involve the recording of perinal striated muscle activity. Rises in corpora spongiosum pressure during penile erection are much lower than those seen in the corpora cavernosum. Additionally, such copora spongiosum rises are associated with contraction of the bulbospongiosus muscles (Purohit and Beckett, 1976). So, corpora spongiosum erectile tissue may have a more prominent role in ejaculation as opposed to penile erection which helps to explain the preference for using the ICP model when measuring erectile responses.

Apomorphine (Ki=101, 32, 26, 2.6, 10nM for D1, D2, D3, D4 and D5 receptors, and low affinity for adrenergic α and serotonergic receptors) injected i.v. (Giuliano et al, 2001) or directly into the PVN

(Chen et al, 1999) produces transient increases in ICP in the anaesthetised rat. Additionally, administering compounds into the PVN that act to enhance dopaminergic responses have similar effects on ICP (Allard et al, 2002). Thus, these findings add to those behavioural studies confirming a role for dopamine acting in the PVN during the expression of penile erection. The paraventricular target neurons believed to mediate the effects of dopamine remain unknown. However, oxytocin neurons in the PVN are believed to be one potential central substrate. As we have previously shown in Chapter 3, stimulation of D2 and D3 receptors in the brain induces an erectile response in male rats that seems to partly involve the activation of paraventricular parovcellular oxytocin cells. Additionally, oxytocin cells within the pPVN were shown to possess D2, D3 and to a lesser extent D4 receptors (Chapter 4). So, our findings provide some more evidence for a role of the paraventricular oxyocin system in mediating the erectogenic effects of dopamine agonists. Our results also lightly touch on the possibility of a direct dopaminergic influence on this oxytocin population during sexual behaviour.

The PVN contains a heterogenous population of cells and possesses a distinct cyto-architecture. As previously described, paraventricular oxytocin cells can be divided into magnocellular and parvocellular sub-populations. The magnocellular neurons are larger in diameter and project to the neurohypophysis where they release oxytocin and vasopressin into the blood to regulate such functions as milk ejection and parturition (Lin et al 2003; Neumann et al, 2001; Russell et al, 2003). Parvocellular neurons are smaller in diameter and are known to project to extra-hypothalamic sites in the CNS such as the brainstem (Petrov et al, 1995), the cervical-thoracic (Hosoya et al, 1991; Swanson and Kuypers, 1980; Toth et al, 1999) and the lumbo-sacral spinal cord (Veronneau-Longueville et al, 1999; Tang et al, 1998) where they are believed to regulate autonomic processes including cardiovascular and erectile function. Recent findings postulate that parvocellular oxytocin release at these sites contributes to central control of cardiorespiratory activities (Mack et al, 2002; Mack et al, 2007), penile erection and copulation (Argiolas & Melis, 2004 for review; Melis & Argiolas, 2003 for review).

Chapter 5.1: Lumbosacral oxytocin action and apomorphine-induced penile erection - Introduction

5.1.1 Decscending paraventriculospinal pathway

Since dopaminergic activation of the PVN elicits penile erection (Melis et al, 1987) and the PVN contributes oxytocin fibres to the lumbosacral spinal cord (Veronneau-Longueville et al, 1999) where local oxytocin action potently induces erectile function (Giuliano et al, 2001); it has been inferred that initiation of penile erection is preceded by the activation of a descending paraventriculospinal oxytocin pathway. Dopamine is thought to act on cell bodies of parvocellular oxytocin neurons via dopamine receptors to stimulate release of oxytocin at the lumbosacral spinal cord and facilitate penile erection. However, currently there is no direct evidence to show that dopamine activates this pathway or can alter oxytocin concentration in the spinal cord of the male rat during penile erection.

An increase in oxytocin concentrations in the CSF of copulating male rats was first demonstrated by Hughes and colleagues (1987). Since then there have been a growing number of studies implicating oxytocin action in the lumbosacral spinal cord as an important mediator in the generation of penile erection. The erection generator network receives oxytocinergic innervation and possesses oxytocin receptor binding sites (Tang et al, 1998; Veronneau-Longueville et al, 1999). Additionally, local injection of oxytocin into the lumbosacral spinal cord strongly induces ICP rises (Giuliano et al, 2001). These findings suggest that oxytocin can activate spinal networks and in turn elicit penile erection. The central stimulus/stimuli which provide supraspinal command and so partly influence the activation of the spinal cord-projecting oxytocin fibres are far from being elucidated. However, increased dopaminergic activity in the PVN is emerging as one potential excitatory input to oxytocin neurons that may contribute to the initiation of penile erection. It has been hypothesised that spinal oxytocin release may be partly influenced by dopaminergic stimulation at the paraventricular level.

5.1.2. Anatomical evidence

Tracer and immunocytochemical studies have revealed that parvocellular oxytocin neurons, originating in the PVN, project to many spinal sites such as the superior cervical ganglion

(Teclemariam-Mesbah et al, 1997), the intermediolateral column and the dorsal vagal complex (Swanson & Sawchenko, 1980), the dorsal grey commisure (DGC) of the thoracolumbar (sympathetic) and lumbosacral (parasympathetic) spinal cord (Swanson and McKellar, 1979). More specifically, parvocellular neurons in the anterior, dorsal and medial pPVN are known to terminate in the L5-L6 area of the spinal cord which contains the sexually dimorphic spinal nucleus of the bulbocavernosus (Wagner and Clemens, 1991); a motor nucleus which innervates the perineal muscles involved in penile reflexes. Additionally, in the L4-L6 spinal cord, some oxytocin fibers have been shown to make direct synaptic contacts with preganglionic neurons in the sacral parasympathetic nucleus (SPN); a pro-erectile spinal nucleus believed to be an integration site, co-ordinating supraspinal commands and peripheral output to the penis (Swanson & McKellar, 1979; Tang et al, 1998; Veronneau-Longueville et al, 1999). It is possible that these oxytocin fibres may also act on interneurons in the DGC or SPN (Tang et al, 1998). Additionally, oxytocin binding sites have been located in the dorsal horn, DGC and SPN of the rat lumbosacral spinal cord (Veronneau-Longueville et al, 1999). Such neuroanatomical evidence suggests that there is a strong overlap between oxytocinergic paraventriculo-spinal neuronal projections and the distribution of oxytocin binding sites in the lumbosacral spinal cord. Impinging on these descending paraventricular oxytocin fibres are incertohypothalamic-derived dopaminergic fibres (Buijs et al, 1984; Lindvall et al, 1984). Therefore, there is a strong basis for the existence of a descending oxytocinergic parvocellular pathway of paraventricular origin which terminates in the lumbosacral spinal cord and is innervated by branching dopaminergic fibres.

5.1.3. Lesion studies

Lesions which destroy the descending parvocellular fibres to the lumbosacral spinal cord have been shown to deplete almost all (75%) oxytocin concentrations in the CSF (Hawthorn et al, 1985) and disrupt oxytocin-like immunoreactivity in the lumbosacral spinal cord (Ackerman et al, 1997; Monaghan et al, 1993). Damage to the whole PVN (thus disrupting magnocellular and parvocellular regions) does not cause any apparent deficits in penile erection but can affect some copulatory

parameters (Liu et al, 1997). In addition, local PVN lesions which selectively destroy spinal cord-projecting neurons resulted in an increase in the latency and a decrease in the frequency of non-contact erections suggesting a specific effect on the activation of pathways (presumably parvocellular) regulating erection. Furthermore, in addition to general parvocellular involvement in erectile function, it seems that specific parvocellular subregions in the PVN may differentially modulate certain parameters of sexual behaviour. Local lesioning of the lateral and posterior parvocellular parts of the PVN completely abolishes the ejaculation-related increase in CSF oxytocin concentration (Hughes et al, 1987). The particular importance of paraventricular oxytocin neurons in the mediation of dopamine-induced penile erection was demonstrated in another lesioning study where penile erection elicited by apomorphine was profoundly inhibited after destruction of the PVN (Argiolas et al, 1987). This strongly suggests that the descending paraventriculospinal oxytocin pathway is intergral to the pro-erectile actions of dopamine in particular. Thus, whilst it is not possible to delineate which oxytocin phenotype in the PVN is excited by apomorphine, there is some convincing evidence which supports a role for parvocellular oxytocin neurons (projecting to the lumbosacral spinal cord) in the initiation of penile erection evoked by remote oestrus female cues. Conversely, magnocellular oxytocin neurons may have more of a modulatory role in reflexive erections and influence those sexual events occurring after the achievement of penile erection eg. intromission and/or ejaculation.

5.1.4. Neuronal activation studies

Immunocytochemistry studies using Fos protein as a marker of neuronal activation have similarly implicated involvement of parvocellular oxytocin cells in the PVN during penile erection. We have previously shown that oxytocin cells in the medial parvocellular region of the PVN are activated upon Quinelorane-induced penile erection (Chapter 2, section 2.3.1.3.). In addition, oxytocin-induced penile erections significantly induce Fos expression in the dorsal, medial and lateral subregions of the PVN (Kita et al, 2006). Finally, an increase in Fos expression in lateral parvocellular oxytocin cells was observed during intromission and ejaculation (Witt & Insel, 1997).

In conclusion, neuronanatomical, pharmacological and immunocytochemical evidence have revealed that paraventricular descending oxytocinergic neurons serve as one candidate pathway important for generating penile erection. However, whether disruptions in this pathway can affect dopamine-induced penile erection or whether dopamine can stimulate release of oxytocin in the lumbosacral spinal cord is not yet known. Therefore, in this study, we aimed to test the hypothesis that dopamine-induced penile erection involves oxytocin release in the lumbosacral spinal cord.

Chapter 5

5.2 Methods

5.2.1. Effects of an oxytocin receptor antagonist (i.s.) on apomorphine-induced ICP rises

5.2.1.1. Animals

Naïve Sprague-Dawley male rats (200-300g) were used and maintained throughout on a normal 12:12h light dark cycle (lights on from 07:00am to 7:00pm). The rats were housed in groups of 4-5 and were allowed access to food and water *ad libitum*. All experiments conformed to the UK Animals (Scientific Procedures) Act 1986.

5.2.1.2. Drugs

Apomorphine (Sigma, UK) was dissolved in isotonic saline containing 0.1% ascorbic acid (Sigma, UK) and then diluted further in isotonic saline prior to injection. Oxytocin (Sigma, UK) or the oxytocin receptor antagonist, UK-427,843 (developed by Pfizer Labs, Sandwich,UK) were both dissolved in saline prior to administration.

5.2.1.3. Anaesthesia and vascular cannulae

As described in the General Methods, rats were anaesthetised with 1.2g/kg urethane (i.p. Sigma, UK) and placed on a homeothermic blanket to maintain body temperature during the experimental test period. The femoral artery and femoral vein were both cannulated (Portex Ltd., UK) to measure blood pressure and allow drug delivery.

5.2.1.4 Recording of ICP and blood pressure (BP)

For recording of ICP, the penis was denuded of skin and all connective tissue removed. A 25-gauge needle attached to a catheter and connected to a pressure transducer (EM 750, Elcomatic, Glasgow, UK) was inserted into one corpus cavernosum.

Blood pressure was recorded simultaneously because changes in blood pressure can markedly affect blood supply to the penis and so influence ICP responses (Giuliano et la, 1993). As explained in General Methods (section 8.1.3.), the amplitude of each ICP rise was thus standardised according to the corresponding mean BP. Recording of BP was performed as described in General Methods (section 4.1.4.).

5.2.1.5 Intraspinal injections

For intraspinal (i.s.) injections, rats were initially placed in their ventral surface and a small opening was made between the T13 and L1 vertebrae to expose the L4-L6 spinal cord. Using a 10µl Hamilton syringe (Hamilton, Switzerland) secured in a stereotaxic frame, the needle was lowered into the lumbosacral spinal cord, lateral to the central vein and approximately 1-1.5mm deep. Drugs were administered in 3µl over 3 min by hand.

5.2.1.6 Intra-PVN injections

For intra-PVN injections, rats were secured in the stereotaxic frame. Checks were made to ensure lambda and bregma were level before aiming a 10µl Hamilton injection syringe at the PVN (stereotaxic co-ordinates; 1.8mm caudal, 0.5mm lateral (from bregma and 8.6mm ventral from the skull surface). Drugs were injected in 2µl over 2 min by hand.

For all drug injections using the 10µl Hamilton syringe, the injection needle was kept in place for a further 30 secs to ensure diffusion of the drug from the needle and to prevent backflow of the drug into the injection needle.

5.2.1.7 Histology and location of injection sites

At completion of the experiment, rats were given an overdose of urethane. The brain or L4-L6 region of the spinal cord were then dissected out and stored in formalin (with 4% paraformaldehyde) for 24 hours before being cut on a cryostat (30µm). Brain sections containing the PVN or L4-L6 spinal cord sections were mounted onto gelatinised microscope slides. Sections are then firstly placed in decreasing ethanol concentrations (100% x2, 90%, 70%. 50%x2) followed by methylene blue and then dehydrated in increasing ethanol concentrations (50% x2, 70%, 90%, 100% x2) and histoclear x2 for 2 min each prior to coverslipping. The site of injection was located using a light microscope at x20 magnification.

5.2.1.8 Experimental design

Initially, we aimed to establish the erectile response to intra-PVN delivery of apomorphine. Rats were injected with either vehicle (saline supplemented with 0.1% ascorbic acid) or 1µg of apomorphine (4µl) in the PVN (rationale for injecting such a large volume is explained in section 5.4.1.1) to confirm that apomorphine delivered intra-PVN can induce increases in ICP in the anesthetised rat.

We then proceeded to establish the erectile dose-response to intravenous (i.v.) delivery of apomorphine. Seven groups, each of 5 rats were injected with either vehicle (saline supplemented with 0.1% ascorbic acid) or 0.1, 0.5, 0.15, 0.25, 1 or 5 mg/kg apomorphine i.v. This experiment allowed the determination of a sub-maximal effective dose of apomorphine to be used in subsequent experiments.

Following these, the ICP response to i.s. delivery of oxytocin was assessed. One group of 5 rats was injected with oxytocin (30ng in 3µl). This experiment was designed to mimic release of oxytocin in the lumbosacral spinal cord and show that oxytocin injected at L4-L6 spinal level is effective in eliciting penile erections. This therefore confirmed the correct injection site for the oxytocin receptor antagonist.

After the above preliminary studies, the dose-dependent inhibitory effect of the oxytocin antagonist UK-427,843 on apomorphine-induced penile erections was assessed. Four groups, each of 6-7 rats were injected with vehicle (saline) or 36, 180 or 900 ng of UK-427843 i.s. (3µl). The injection in the spinal cord was performed 5 min prior to i.v. administration of apomorphine. This experiment allowed direct testing of the ability of an oxytocin antagonist delivered at the lumbosacral spinal cord to inhibit penile erections induced by apomorphine injected i.v.

In all ICP studies, basal ICP conditions were recorded for 10 min prior to apomorphine (or oxytocin injection) and then observed for a further 30 min after apomorphine (or oxytocin / oxytocin antagonist injection).

5.2.1.9 Quantification of ICP responses

Only ICP rises with a maximal value greater than the sum of the average + 2 standard deviations of the ICP recording during 10 min prior to drug injection were included in analyses. The following parameters were measured:

- Incidence (%) of erectile responses (number of rats showing at least 1 ICP rise)
- Mean number of erectile responses per rat

- Latency to the first ICP response
- Duration of ICP response
- ICPmax/BP (maximum amplitude of ICP rise normalised according to corresponding BP)
- SUM AUC/BP (overall duration of ICP rise, considering the amplitude and duration of the ICP rise. As above, values are standardised according to the corresponding BP).

All of the above measured parameters were averaged per group, except the SUM AUC/BP. Those rats who did not show any ICP responses within the 30 min test period, were assigned a value of zero for the incidence, mean number of ICP increases and AUC/BP. Additionally, when calculating the ICPmax/BP, AUC/BP, latency and duration, those rats who did not elicit at least one ICP response in the 30 min experimental test period were excluded from statistical analysis.

5.2.1.10 Statistics

The different treatments within each experiment were randomised as necessary. Data to be analysed were the incidence of ICP rises (%) (Chi-squared test), the mean number of erectile responses, latency, duration, ICPmax/BP and the SUM AUC/BP (all parameters were assessed using One Way ANOVA followed by Dunn's method). P values <0.05 were considered statistically significant.

5.2.2. Effect of apomorphine on oxytocin release in the lumbosacral spinal cord

5.2.2.1. Animals

Due to financial and time constraints, naïve Sprague-Dawley male rats (200-250g) were used in this study and maintained throughout on a normal 12:12h light dark cycle (lights on from 07:00am to

7:00pm). The rats were housed in groups of 5 and were allowed access to food and water *ad libitum*. All experiments conformed to the UK Animals (Scientific Procedures) Act 1986.

5.2.2.2. Drugs

Apomorphine (Sigma, UK) was dissolved in 0.1% ascorbic acid (Sigma, UK) and then diluted in saline.

5.2.2.3. Surgical preparation

Rats were anaesthetised as before and cannulation of the femoral artery, L4-L6 spinal cord dissection and histology were performed as previously described in General Methods and section 4.2.1.3. Because penile erection was not recorded in this study, we did not measure blood pressure in these animals.

5.2.2.4. Microdialysis

As described in the General Methods, the T13-L1 vertebrae were mounted on the stereotaxic frame to expose the L4-L6 spinal cord. A microdialysis probe (Brain microdialysis probe, 2mm membrane, Bioanalytical Systems, Inc, Indiana, USA) was secured in a probe holder in the stereotaxic frame. The microdialysis probe was connected via teflon tubing (FEP tubing, 50cm in length, 6µl in volume, Linton Instrumentation, UK) to a 100µl Hamilton syringe mounted on a "push" perfusion pump (PHD 2000 Infuse/Withdraw pump (Harvard Apparatus, USA) set at a flow rate of approximately 3µl/min. The probe was perfused with artificial cerebrospinal fluid (ACSF) and positioned in to the lumbosacral spinal cord as described before. After an initial 30 min ACSF wash, two 30 min baseline samples were collected. Apomorphine (0.25mg/kg) was then injected at 0 min and a further three 30 min analyte samples were collected at 30, 60 and 90 min after which time the animal was sacrificed

with an overdose of urethane anaesthesia. All samples were collected in sterile eppendorfs containing 0.2M HCl (10µl) and placed in dry ice before transferring to -20°C.

4.2.2.5. Push-pull perfusion

The animal was secured in the stereotaxic frame as described above and a microdialysis probe (as above) lacking the 2mm membrane was used as a push-pull probe. The probe was connected to both a "push" pump (set at 10-11µl/min) and a "pull" pump (Miniplus 3 peristaltic pump, Gilson, France) via teflon tubing (FEP tubing, 25cm in length, 3µl in volume, Linton Instrumentation, UK) , set at 0.55 arbitary units (resulted in the collection of 10-11µl samples per min). The "push" and "pull" pumps were calibrated prior to and after insertion of the probe into the lumbosacral spinal tissue to ensure the sample collection rats were maintained at 10-11µl/min. The sample collection protocol for push-pull perfusion was the same as that for microdialysis.

5.2.2.6. Recording of breathing frequency

Apomorphine can act as a cardiovascular depressant and affect breathing frequency, which can cause slight movement around the probe injection site. Due to such movement during breathing, local tissue damage around the probe site may have some effect on oxytocin release in the lumbosacral spinal cord. Thus, in order to ensure that any observed changes in oxytocin levels were predominantly due to the effects of apomorphine, the number of inspirations in 60 secs was recorded at the following intervals for each rat; -30, -5 and 5 min. An inspiration was defined as a single visible expansion of the thoraco-abdominal cavity in the anaesthetised rat.

5.2.2.7. Oxytocin radioimmunoassay

Oxytocin radioimmunoassay was perfomed by Rainer Landgraf (Max Planck Institute of Psychiatry, Munich). Details are previously described in Chapter 2 (section 2.11.2).

5.2.2.8. Experimental design

This experiment was designed to assess the effects of apomorphine (0.25mg/kg) (i.v.) on oxytocin release in the lumbosacral spinal cord of the anaesthetised rat. For microdialysis, 5 rats were initially injected with i.v. apomorphine. For push-pull perfusion, rats were injected i.v. with apomorphine (n=8) or vehicle (saline supplemented with 0.1% ascorbic acid) (n=6).

5.2.2.9. Statistics

Those rats that died during the experimental test period (5/13) due to them encountering breathing difficulties were excluded from statistical analysis. Differences in oxytocin levels between vehicle and apomorphine treated rats at -60, -30, 0, 30, 60, 90 min were compared (Two Way ANOVA with repeated measures). Differences in oxytocin levels in apomorphine treated rats at -60 (basal) and 30 min were also assessed (one way ANOVA for repeated measures). Delta values (oxytocin concentration at 30 min minus mean basal concentration) were calculated for both vehicle and apomorphine treated rats and statistical comparisons in the overall oxytocin response were analysed (Unpaired Student's t-test). Finally, the effects of apomorphine on breathing frequency were also assessed (Unpaired Student's t-test). P values <0.05 were considered statistically significant.

Chapter 5

5.3 Results

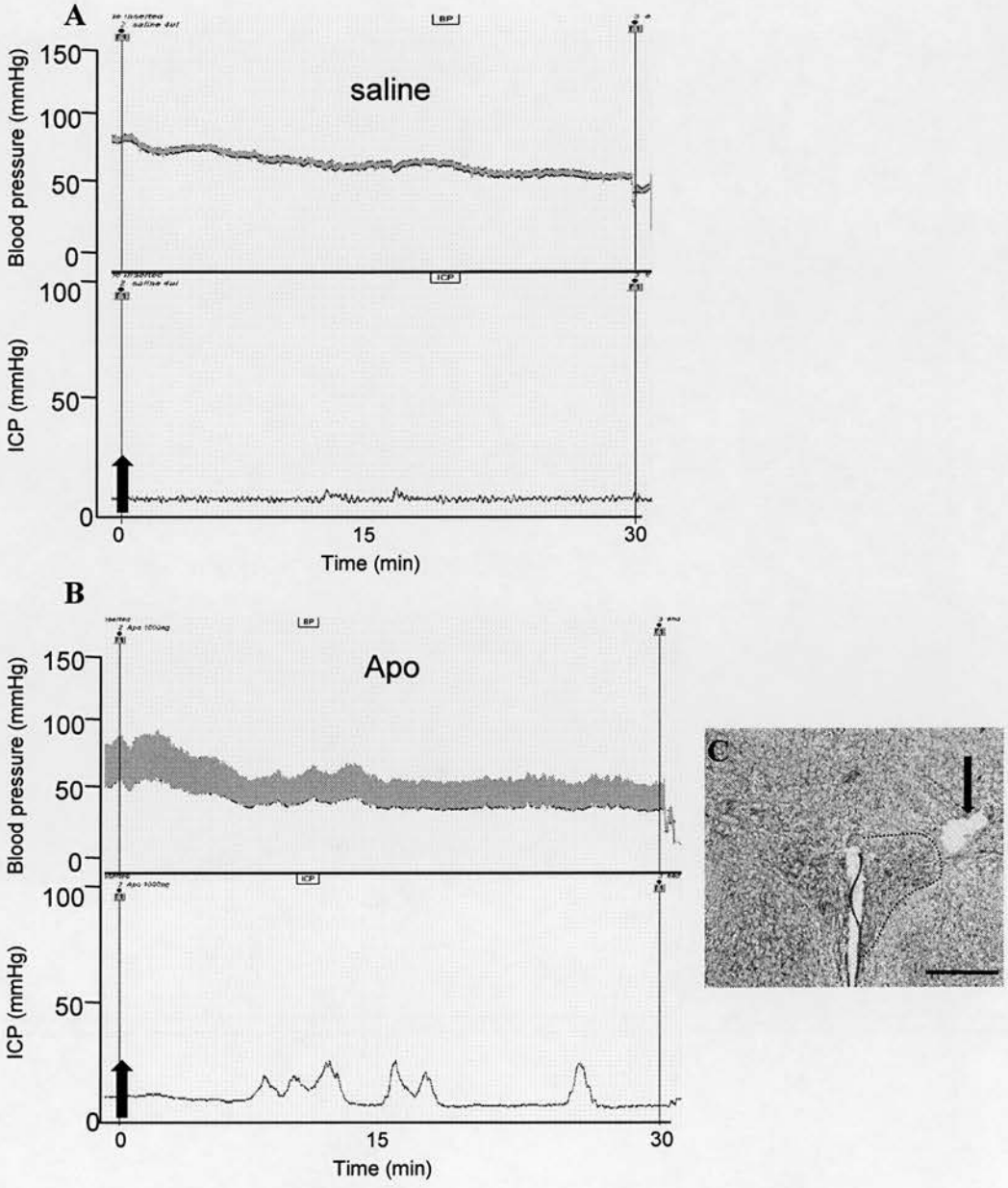
5.3.1. *Effects of an oxytocin receptor antagonist on apomorphine-induced ICP rises*

5.3.1.1. Erectile dose response curve to apomorphine (intra-PVN)

Because we wanted to selectively activate the PVN and measure erectile responses an injection needle delivering apomorphine was stereotaxically aimed at the PVN. Figure 5.1A and 5.1B show the example ICP responses to intra-PVN delivery of vehicle and apomorphine (1μg in 4μl), respectively. Intra-PVN injection of vehicle (5.1A) elicited only marginal increases in ICP; such small rises are often termed "spontaneous ICP rises" and have been reported before in studies using the ICP model (Giuliano et al, 2001). These ICP rises are not necessarily related to intra-PVN injection of vehicle (saline + 0.1% ascorbic acid) but reflect the innate erectile responses that can occur under any experimental recording conditions. Indeed in conscious male rats, spontaneous erections (reflected by spontaneous ICP rises in the anaesthetised rat) are frequently observed in vehicle groups (Melis et al, 2005, 2006).

Intra-PVN injection of apomorphine (5.1B) produced transient increases in ICP. Apomorphine increased the number of ICP rises and each peak was of higher amplitude and longer duration compared to those ICP rises elicited in control animals. The photomicrograph in 5.1C illustrates the injection site histologically. It can be seen that the injection needle reached the very lateral region of the mPVN. Unfortunately, direct injection in the PVN proved technically very difficult and the success rate was relatively low, thus the number of rats per group was low. Thus, statistics could not be performed on this set of data. To minimise the number of animals used and due to time restrictions, it was decided to administer apomorphine i.v. for all subsequent studies.

Figure 5.1: Effects of apomorphine (intra-PVN) on ICP rises



Effects of central injection (which lies very close to the PVN) of apomorphine (Apo, 1μg) on ICP. Example traces show blood pressure (top) and ICP responses (bottom) to central delivery (in close proximity to the PVN) of vehicle (A) and Apo (B) . The photomicrograph in C illustrates the injection site (black arrow). Apo induces transient increases in ICP compared to vehicle. Scale bars represent 200μm.

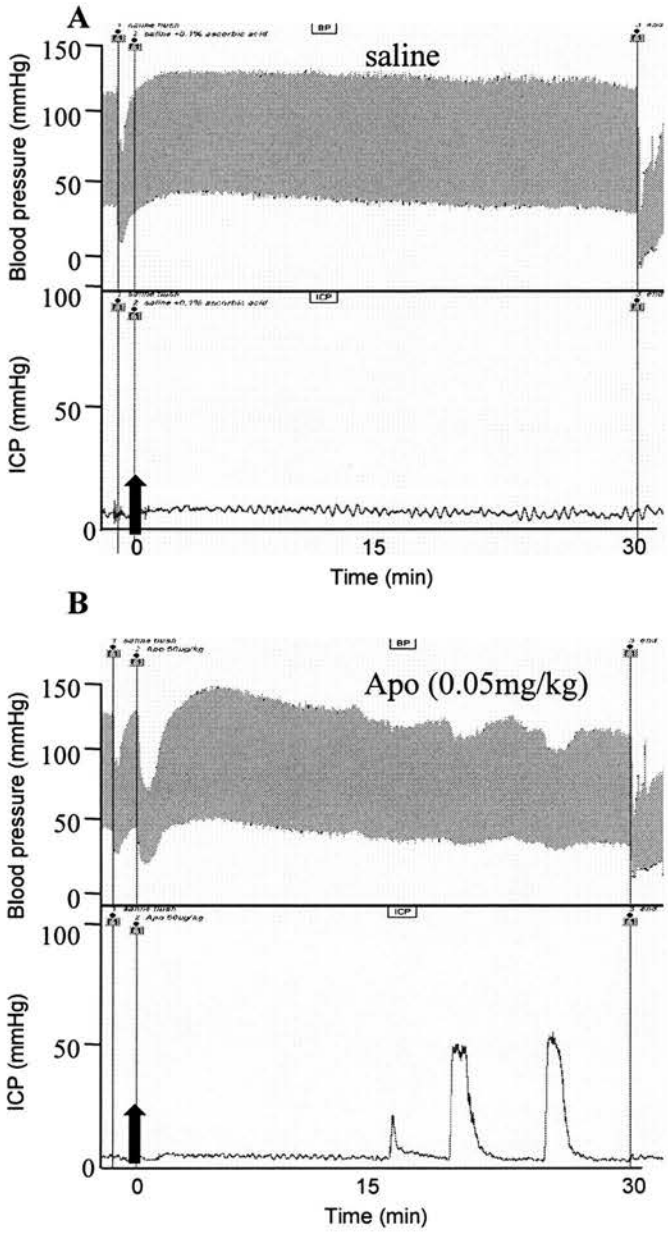
5.3.1.2. Erectile dose response curve to apomorphine (i.v.)

Apomorphine (i.v.) (0.01, 0.05, 0.15, 0.25, 1, 5mg/kg) dose-dependently induced transient increases in the ICP baseline. Figures 5.2-5.4 show example traces of rats treated with vehicle (Figure 5.2A), 0.05 (Figure 5.2B), 0.25 (Figure 5.3B) or 1mg/kg (Figure 5.4B). In each trace, a transient decrease in blood pressure can be observed after each saline flush and prior to apomorphine injection. This is due to the presence of urethane in the catheter prior to the saline flush. It is well known that urethane can have a depressant effect on sympathetic neuronal firing (Sun and Reis, 1995) and thus explains the momentary fall in systemic blood pressure.

Rats treated with vehicle (i.v.) did not display any spontaneous rises in ICP. As expected, increasing the dose of apomorphine increased the number, amplitude and duration of ICP rises. Since no rats in the vehicle group displayed any ICP rises, amplitude and duration of ICP rises could not be compared statistically. Figure 5.5 shows that apomorphine dose-dependently increased the mean number of ICP rises per rat (Figure 5.5A) (One Way ANOVA followed by Dunn's method) with the 1mg/kg eliciting the greatest number of ICP rises. Apomorphine also increased the incidence (percentage) of rats displaying ICP rises (Figure 5.5B) (Chi-squared test) with 1mg/kg dose being the most effective. From these data, a dose of 0.25mg/kg apomorphine was used in all subsequent studies.

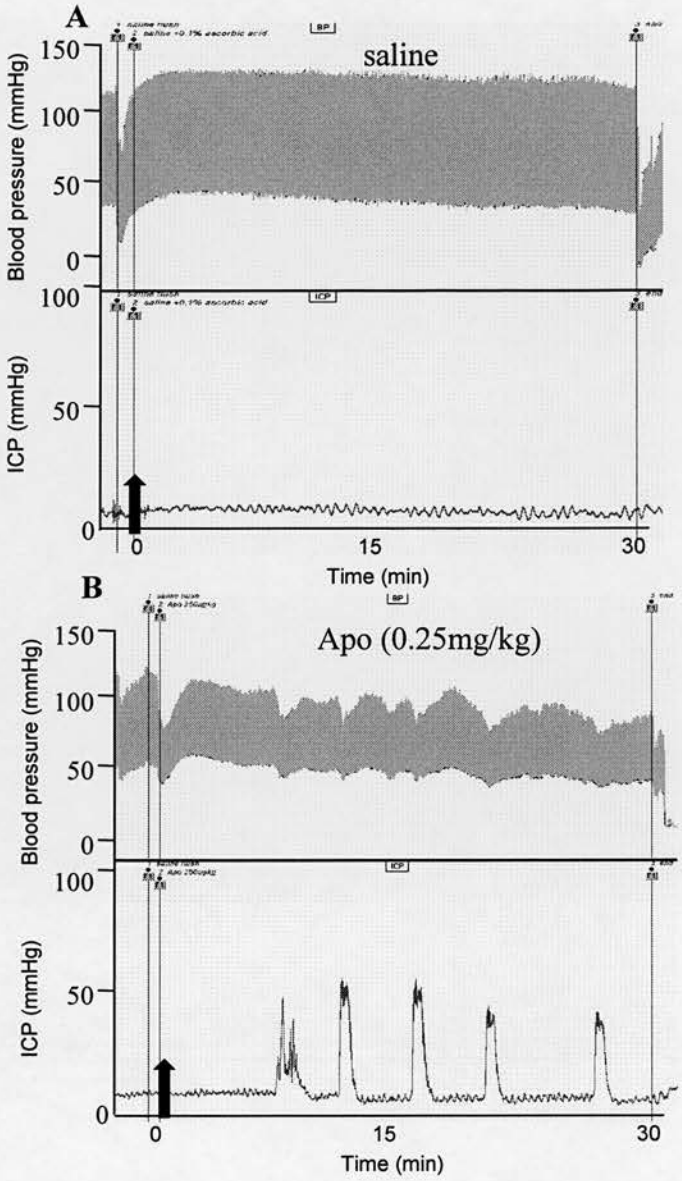
Apomorphine (i.v.) at low doses exhibited a slight effect on systemic blood pressure. It can be seen that the 0.05 and 0.15mg/kg apomorphine significantly decreased blood pressure (Figure 5.6) (One Way ANOVA followed by Dunn's method). However, no other doses of apomorphine had any significant depressant effects on blood pressure.

Figure 5.2: Effects of apomorphine (i.v.) on ICP rises (1)



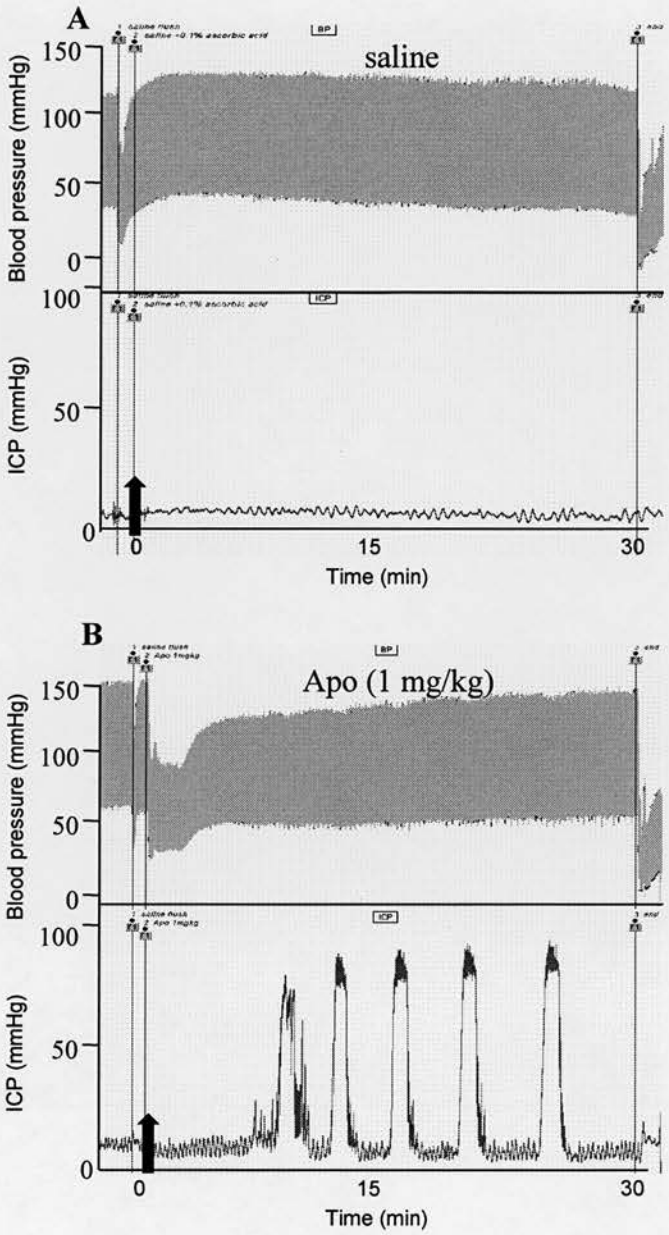
Effects of i.v. delivery of apomorphine (Apo, 0.05mg/kg) on ICP. Example traces show ICP responses to i.v. delivery of vehicle (A) and Apo (B). Apo induces transient increases in ICP compared to vehicle.

Figure 5.3: Effects of apomorphine (i.v.) on ICP rises (2)



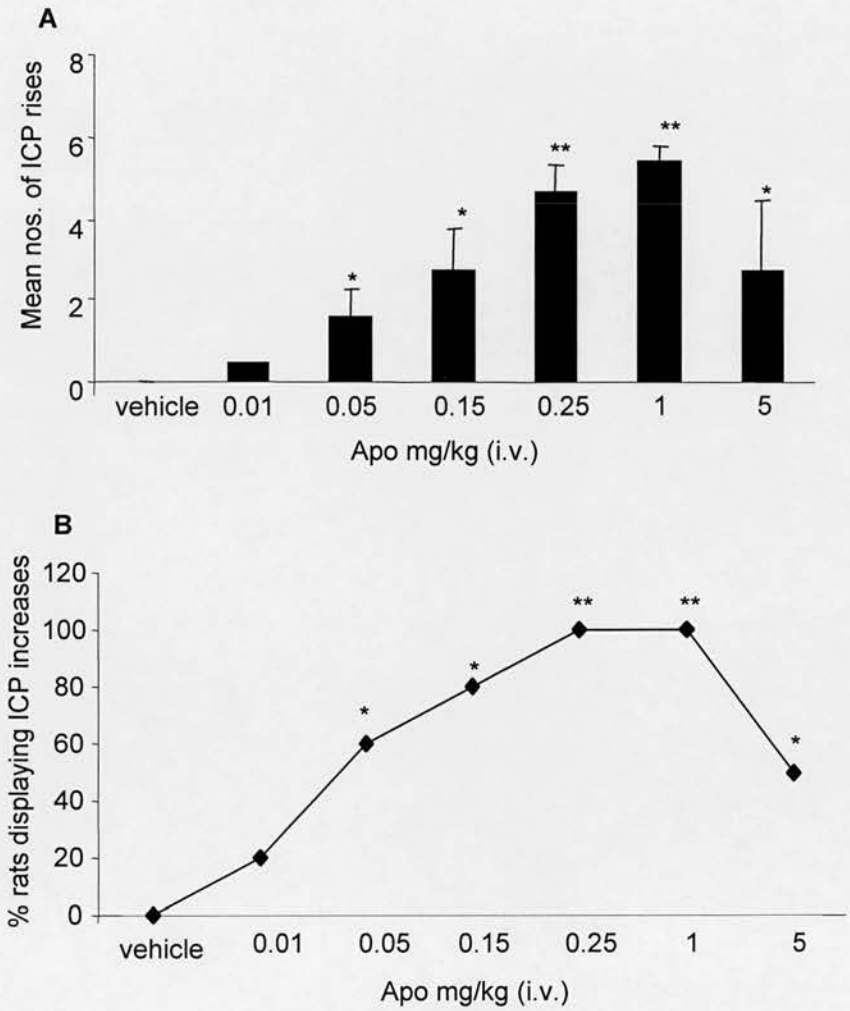
Effects of i.v. delivery of apomorphine (Apo, 0.25mg/kg) on ICP. Example traces show ICP responses to i.v. delivery of vehicle (A) and 0.25mg/kg Apo (B). Apo induces transient increases in ICP compared to vehicle.

Figure 5.4: Effects of apomorphine (i.v.) on ICP rises (3)



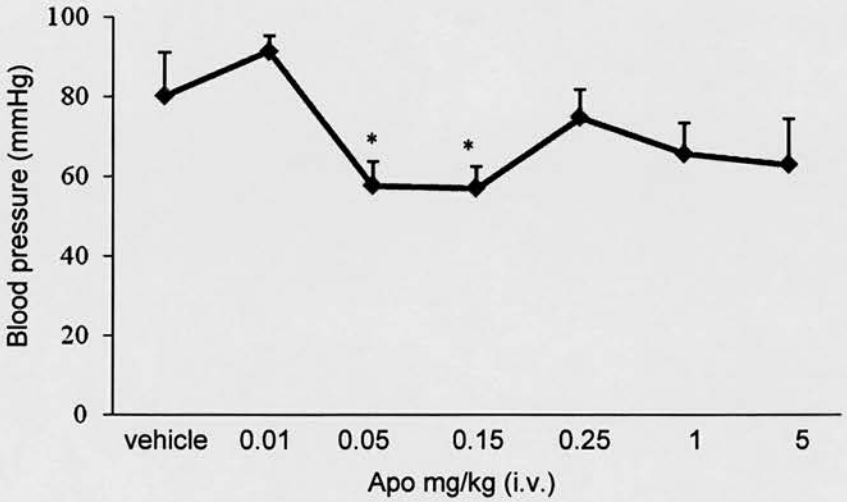
Effects of i.v. delivery of apomorphine (Apo) on ICP. Example traces show ICP responses to i.v. delivery of vehicle (A) and 1mg/kg Apo (B). Increasing the dose of Apo transiently increases ICP.

Figure 5.5: Effects of apomorphine (i.v.) on ICP rises (4)



Effect of apomorphine (Apo) on the mean number of ICP rises (A) and the incidence (%) of penile erection (B). Apo (n=4-5 per group) dose dependently increased the mean number of ICP rises (One Way ANOVA followed by Dunn's method, *=P<0.05; **=P<0.01,) and the percentage of rats displaying ICP rises (Chi-squared test, *=P<0.05; **=P<0.01).

Figure 5.6: Effects of apomorphine (i.v.) on blood pressure



Effect of apomorphine (Apo) on blood pressure in the anaesthetised rat. In those rats treated with Apo only (n=4-5 per group), 0.05 and 0.15mg/kg Apo significantly decreased blood pressure (One Way ANOVA followed by Dunn's method, *P<0.05).

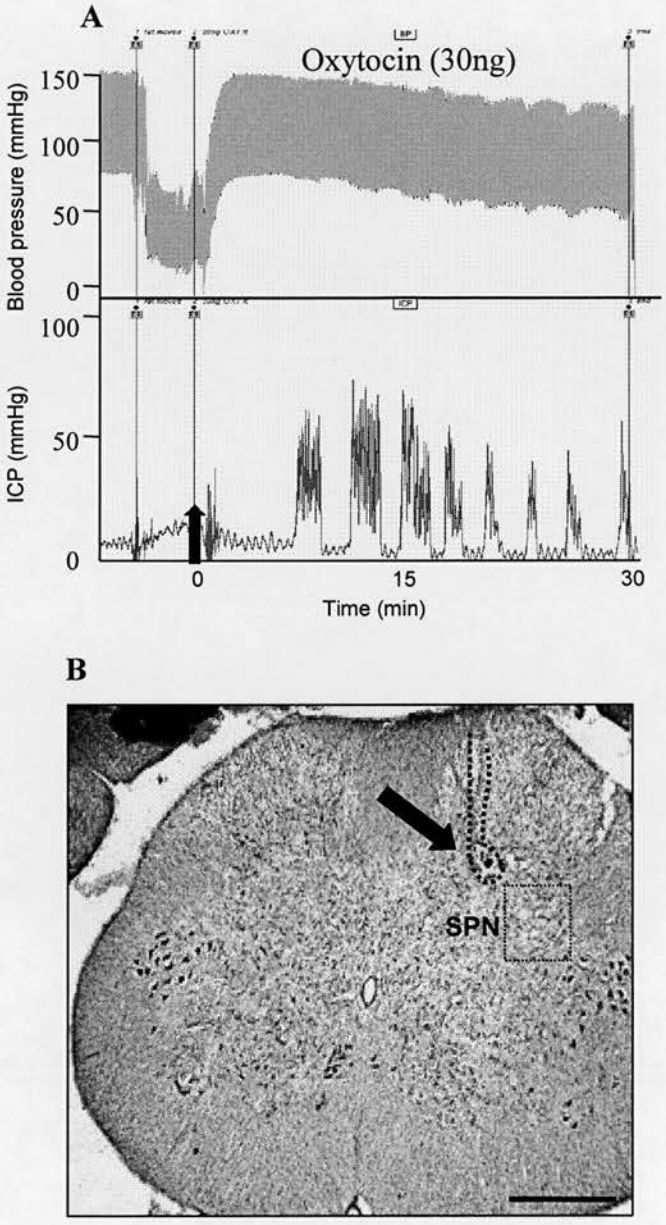
5.3.1.3. Effects of oxytocin (i.s.) on ICP rises

In order to confirm that oxytocin action in the L4-L6 lumbosacral spinal cord elicits rises in ICP, oxytocin (30ng) (n=5) was injected in the L4-L6 spinal level to mimic the release of oxytocin. Typical ICP responses after i.s. injection of oxytocin (30ng) are shown in the example trace in Figure 5.7A. Oxytocin (i.s.) induced robust increases in the ICP baseline in all 5 rats examined. The photomicrograph in Figure 5.7B, histologically shows the injection site. It can seen that the injection needle lies close to the sacral parasympathetic nucleus (SPN) which is an important nucleus involved in the generation of penile erection. As stated previously, i.s. injection of oxytocin in the L4-L6 spinal cord was performed solely to confirm our injection site was in fact the correct spinal level for oxytocin action to produce increases in ICP (this has been shown previously, Giuliano et al 2001). Thus, a vehicle treated group was not included in this part of the study and so statistics were not performed.

5.3.1.4. Effects of an oxytocin receptor antagonist (i.s.) on apomorphine (i.v.)-induced ICP rises

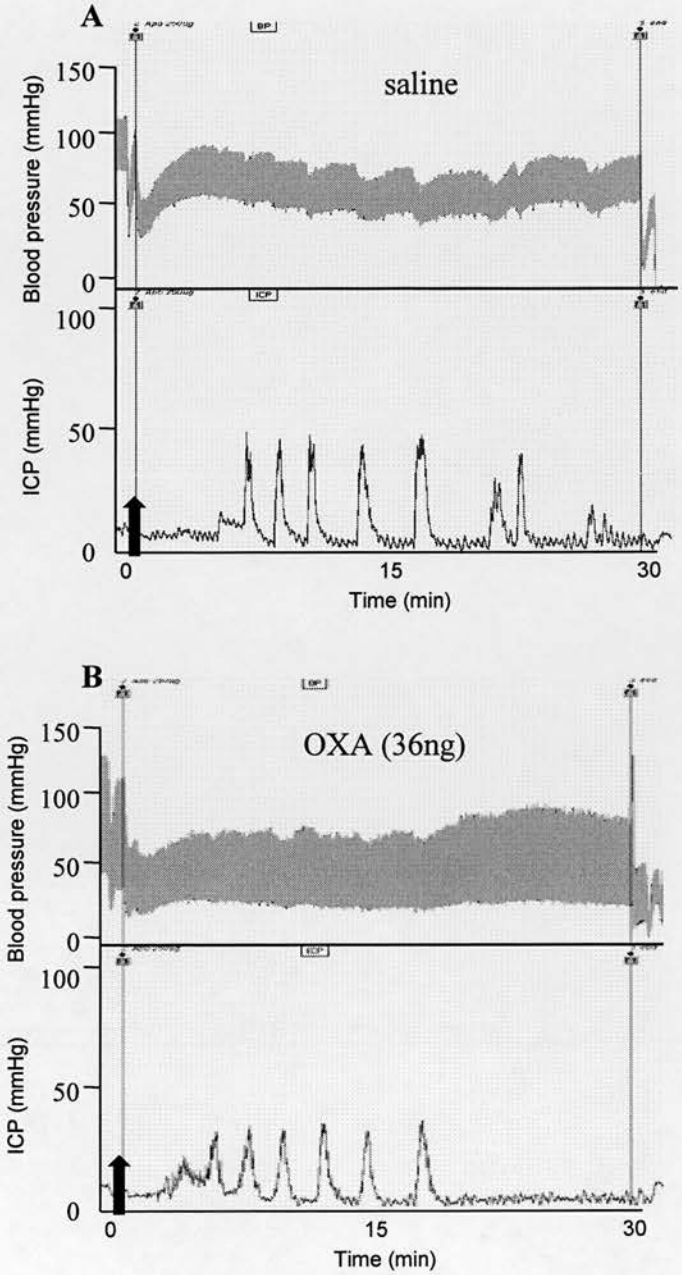
In order to establish that oxytocin action in the L4-L6 spinal cord mediates apomorphine-induced ICP rises, 36, 180 or 900ng of an oxytocin receptor antagonist was injected in the L4-L6 spinal cord 5 min prior to 0.25mg/kg apomorphine (i.v.). Figures 5.8-5.10 show example ICP traces of rats pre-treated with vehicle (Figure 5.8A), 36 (Figure 5.8B), 180 (Figure 5.9B) or 900ng (Figure 5.10B) followed by 0.25mg/kg apomorphine. After quantification it was observed that180 and 900ng of the oxytocin receptor antagonist significantly decreased the mean number of ICP rises per rat (Figure 5.11A) (One Way ANOVA followed by Dunn's method) and the incidence (percentage) of ICP rises (Figure 5.11B) (Chi-squared test) with the dose of 180ng being the most effective in both measured parameters. No significant differences were detected in the latency (Figure 5.11C), duration (Figure 5.12A) or peak amplitude (Figure 5.12B) (ICPmax/BP) of the apomorphine-induced ICP rises after 36, 180 or 900ng of the oxytocin receptor antagonist. The180ng dose of the oxytocin antagonist

Figure 5.7: Effects of oxytocin (i.t.) on ICP rises



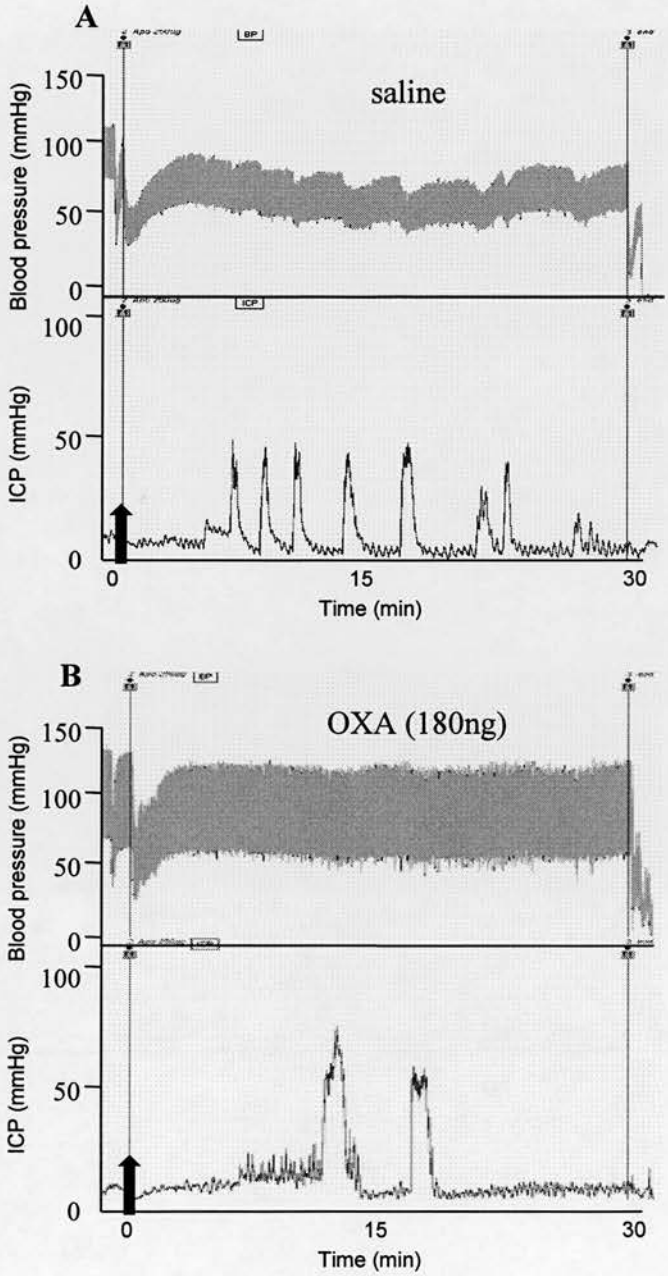
Effect of i.t. delivery of oxytocin (30ng) on ICP. Example trace shown in A displays ICP responses to i.t. injection of oxytocin (30ng). Oxytocin induces increased phasic responses in the ICP baseline. Photomicrograph in B illustrates the injection site (black arrow) in the lumbar spinal cord which lies close to the SPN (hatched box). Scale bars represent 50μm. SPN=sacral parasympathetic nucleus.

Figure 5.8: Effects of an oxytocin receptor antagonist (i.t.) on apomorphine-induced ICP rises (1)



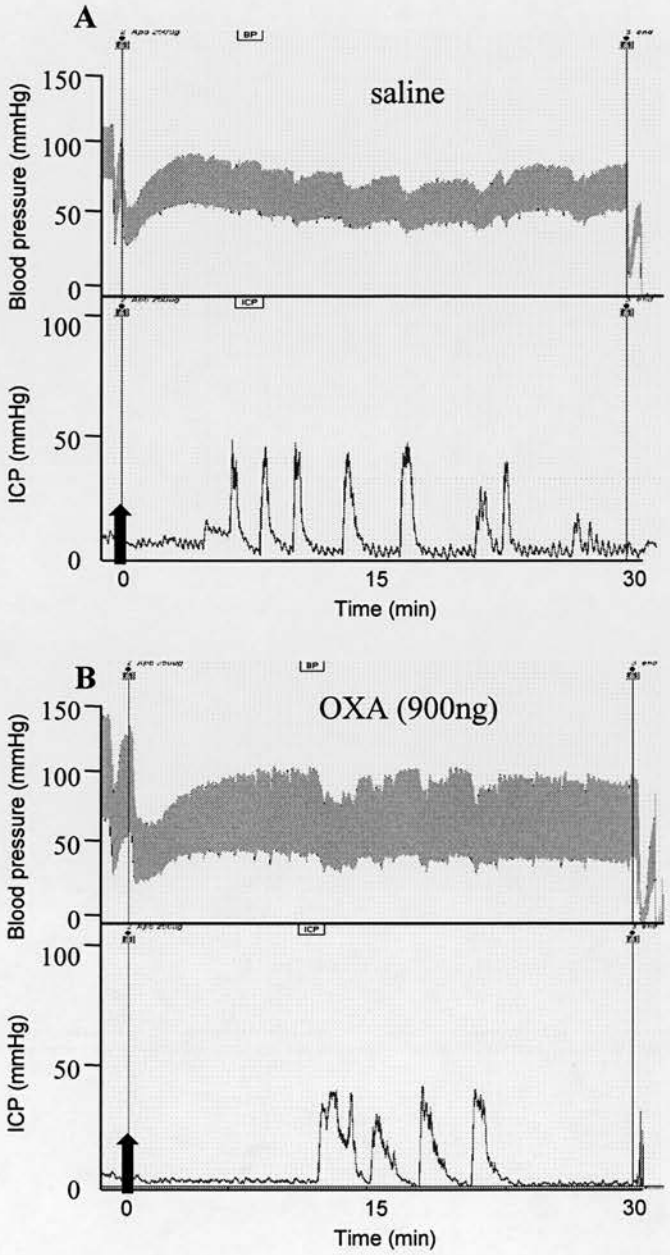
Effect of an oxytocin receptor antagonist (OXA) on apomorphine (Apo)-induced ICP rises. Example traces show ICP responses to i.v. delivery of 0.25mg/kg Apo (as indicated by the black arrow) after i.t. injection of vehicle (A) and 36ng OXA (B). 36ng OXA marginally decreases the frequency of Apo-induced ICP rises.

Figure 5.9: Effects of an oxytocin receptor antagonist (i.t.) on apomorphine-induced ICP rises (2)



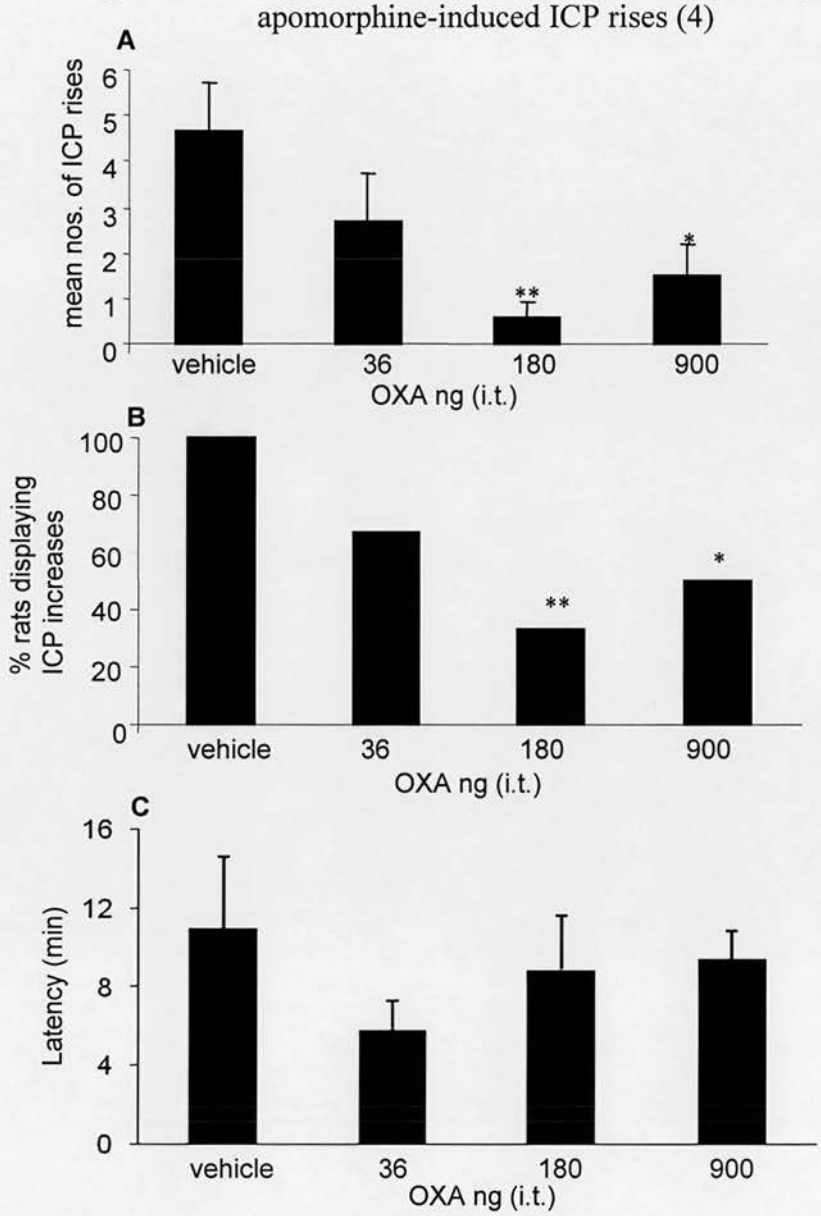
Effect of an oxytocin receptor antagonist (OXA) on apomorphine (Apo)-induced ICP rises. Example traces show ICP responses to i.v. delivery of 0.25mg/kg Apo after i.t. injection of vehicle (A) and 180ng OXA (B). 180ng OXA decreases the frequency of Apo-induced ICP rises.

Figure 5.10: Effects of an oxytocin receptor antagonist (i.t.) on apomorphine-induced ICP rises (3)



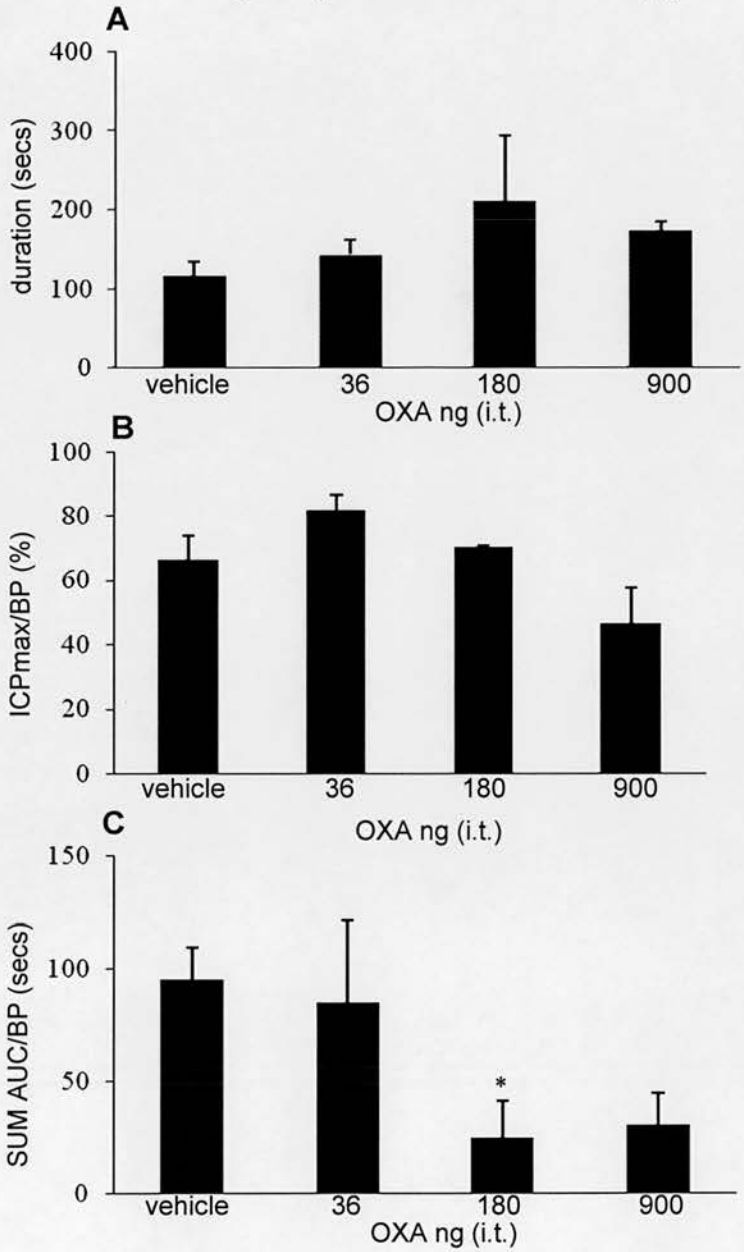
Effect of an oxytocin receptor antagonist (OXA) on apomorphine (Apo)-induced ICP rises. Example traces show ICP responses to i.v. delivery of 0.25mg/kg Apo after i.t. injection of vehicle (A) and 900ng OXA (B). 900ng OXA decreases the frequency of Apo-induced ICP rises.

Figure 5.11: Effects of an oxytocin receptor antagonist (i.t.) on apomorphine-induced ICP rises (4)



Effect of an oxytocin receptor antagonist (OXA) on apomorphine (Apo)-induced ICP rises. The OXA (n=6-7 per group) significantly decreased the mean number of ICP rises (A) (One Way ANOVA followed by Dunn's method, *P<0.05; **P<0.005) and the incidence (%) of ICP rises (B) (Chi-squared test, *P<0.05; **P<0.01). The OXA did not have any significant effect on the latency of Apo-induced ICP increases (C).

Figure 5.12: Effects of an oxytocin receptor antagonist (i.t.) on apomorphine-induced ICP rises (5)



Effect of an oxytocin receptor antagonist (OXA) on apomorphine (Apo)-induced ICP rises. The OXA (n=6-7 per group) did not have significant effect on the duration of ICP rises (A) or the ICPmax/BP (B). Conversely, the OXA (180ng) significantly decreased the SUM AUC/BP (C) (One Way ANOVA followed by Dunn's method, *P<0.05).

(900ng was almost significant P=0.052) significantly reduced the SUM AUC/BP (indicator of overall erectile function) (Figure 5.12C) (One Way ANOVA followed by Dunn's method).

Injection of an oxytocin receptor antagonist on apomorphine-induced ICP rises had no effect on blood pressure as shown in Figure 5.13.

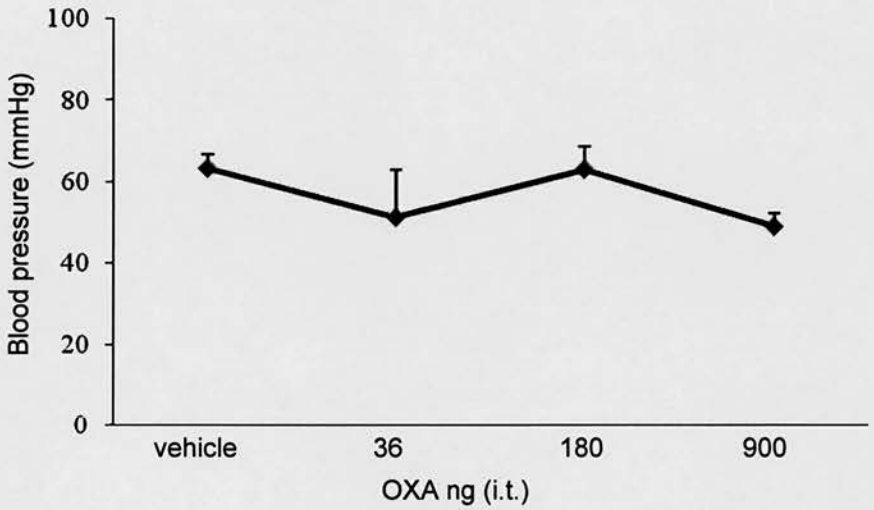
5.3.2 Meausring oxytocin release in the lumbosacral spinal cord after apomorphine (i.v.) injection

5.3.2.1 Effects of apomorphine (i.v.) on oxytocin release in the lumbosacral spinal cord

Because we have shown that lumbosacral oxytocin action partly modulates apomorphine-induced penile erection, we decided to investigate whether apomorphine increases release of oxytocin in the lumbosacral spinal cord. Microdialysis was initially used to obtain samples from the L4-L6 spinal cord. However, as can be seen from Figure 5.14A, the extracted samples contained very low concentrations of oxytocin, perhaps too low to measure any detectable changes after apomorphine injection. Push-pull perfusion yielded samples with greater oxytocin concentrations as can be seen in Figure 5.14B, thus push-pull perfusion was used thereafter.

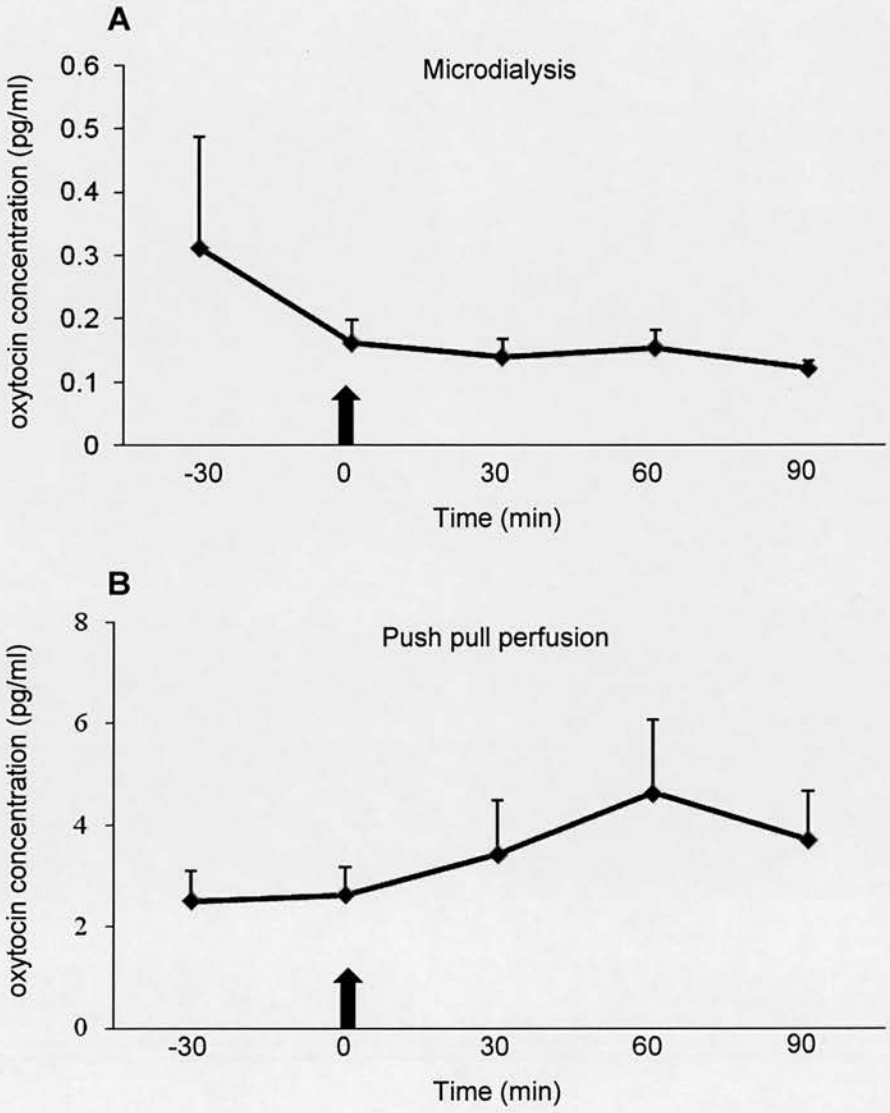
After apomorphine injection at 0 min, there was a non-significant increase in oxytocin release compared to vehicle (Figure 5.15A). Such an increase was not evident at 60 or 90 min. No significant differences were detected when comparing oxytocin levels prior to apomorphine injection with oxytocin concentrations 30 min after apomorphine injection (time period when the largest rise in oxytocin release was detected). Additionally, when comparing the delta values (mean baseline values vs response at 30 min) of vehicle and apomorphine treated rats, those rats receiving 0.25mg/kg apomorphine showed a higher overall oxytocin response compared to vehicle; however this was not significant (Figure 5.15B). One to two rats in the control group did exhibit unexpected high

Figure 5.13: Effects of apomorphine (i.v.) on blood pressure in rats pre-treated with an oxytocin receptor antagonist (i.t.)



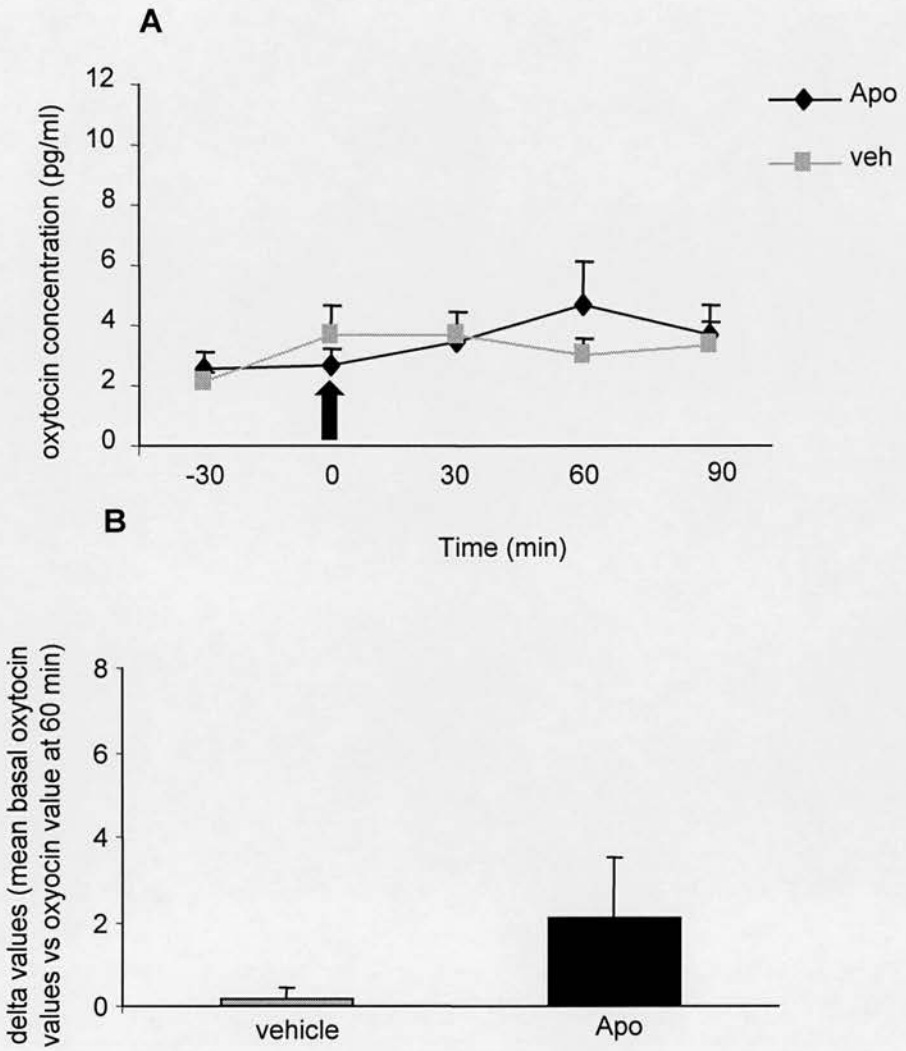
Effect of apomorphine (Apo) on blood pressure in the anaesthetised rat. Rats receiving Apo (0.25mg/kg) after i.t. injection of OXA (n=6-7 per group) did not show any significant depressant effects on blood pressure.

Figure 5.14: Effects of apomorphine (i.v.) on oxytocin release in lumbar spinal cord (1)



Effect of apomorphine (Apo) on oxytocin release in the lumbar spinal cord. Using microdialysis (A) (n=5), no changes in oxytocin baseline were observed after Apo (0.25mg/kg) injection (black arrow) at 0 min. Using push-pull perfusion (B) (n=8), increases in oxytocin release were observed after Apo injection. Additionally, higher concentrations of oxytocin in the collected samples were seen using push-pull perfusion which explains the different scales on the y-axis.

Figure 5.15: Effects of apomorphine (i.v.) on oxytocin release in lumbar spinal cord (2)



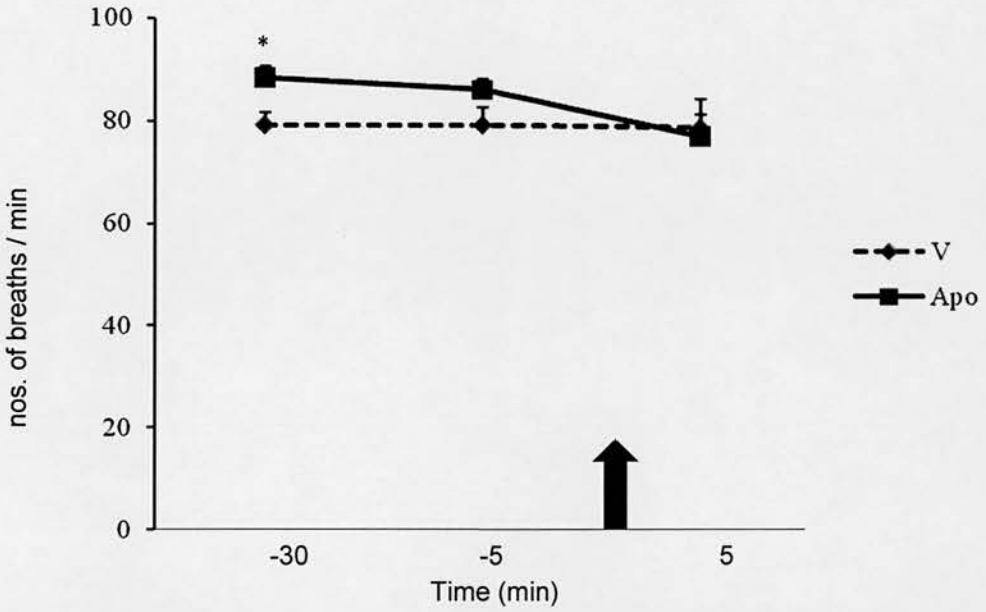
Effect of apomorphine (Apo) on oxytocin release in the lumbar spinal cord. After Apo (0.25mg/kg) injection (black arrow) at 0 min, Apo-treated rats (n=8) showed a non-significant increase in oxytocin release in the lumbar spinal cord compared to vehicle (n=3) (A). When comparing the delta values (mean baseline response vs response at 60 min) of vehicle and Apo, Apo-treated rats produced a greater oxytocin response compared to vehicle; however, this was not significant.

concentrations of oxytocin at time 0 min. The reason for this is unclear but minor technical errors may have been a contributing factor.

5.3.2.2 Effects of apomorphine (i.v.) on breathing frequency

Apomorphine injection at 0 min had no significant effect on breathing frequency in the anaesthetised rat (Figure 5.16). It is interesting to note however, that prior to apomorphine injection, rats placed in the apomorphine-treated group exhibited significantly more inspirations per 60 sec at -30 min when compared to rats in the vehicle group (Figure 5.16) (Two way ANOVA using repeated measures, *$P<0.05$). This was an unexpected finding but perhaps individual variability within each group may account for the levels of significance observed.

Figure 5.16: Effects of apomorphine (i.v.) on breathing frequency in the anaesthetised rat



Effect of apomorphine (Apo) on breathing frequency in the anaesthetised rat. After Apo injection (black arrow), the breathing frequency of Apo-treated rats (n=8) did not significantly differ from vehicle-treated rats (n=6). Prior to Apo injection, those rats placed in the Apo-treated group displayed significantly more breaths per min at 60 min compared to rats placed in the vehicle group (Two way ANOVA using repeated measures, *$P<0.05$).

Chapter 5

5.4 Discussion

5.4.1 Effects of oxytocin receptor blockade in the lumbosacral spinal cord during apomorphine-induced ICP rises

The present study further substantiates previous findings proving apomorphine to be a powerful erectogenic agent in both anaesthetised and conscious animals (Bernabe et al, 1999; Giuliano et al, 2001, 2002; Moreland et al, 2005). Here we have shown that ICP responses induced by systemic injection of apomorphine can be compromised after oxytocin receptor blockade in the lumbar spinal cord. Accordingly, our preliminary results suggest that apomorphine may increase oxytocin release in the lumbar spinal cord to facilitate penile erection. Thus, for the first time we demonstrate a role for spinal oxytocin in the mediation of penile erection upon dopamine stimulation.

5.4.1.1 Effect of apomorphine (intra-PVN) on ICP rises

The PVN has been shown to be one of the most highly sensitive brain regions to respond to the pro-erectile effects of apomorphine (Melis et al, 1987). As expected in this study, intra-PVN (1μg) delivery of apomorphine produced transient increases in ICP which has previously been shown in conscious and anaesthetised animals (Chen et al, 1999; Melis et al, 1987, 2005; Succu et al, 2007). It is noteworthy that the erectogenic doses administered to conscious animals compared to the dose of apomorphine used in this study were comparatively low (100-200ng vs 1μg). A 1μg dose of apomorphine was used because 500ng has previously been known to exert some facilitatory effects on ICP rises (Allard et al, 2002). Thus we used 1μg to almost certainly ensure a pro-erectile response. In addition, general anaesthesia is believed to have some affect on the responsiveness of some neural systems in terminally anaesthetised animals. Thus, a higher dose of apomorphine was injected to compensate for any potential reduction in neuronal excitability due to the anaesthesia. Because it can be technically very difficult (for an inexperienced experimentor) to consistently place the injection

needle in the PVN, apomorphine was given in a 4µl volume (as opposed to 300nl in conscious studies). This larger volume was injected to account for any small stereotaxic injection site inaccuracies (for example, those that lay just outside the PVN), thus increasing the likelihood of diffusion of apomorphine to the PVN. As explained earlier, due to the low success rate of injecting directly in the PVN (as evidenced by an unexpectedly high number of injection tracts located outside the PVN), it was soon decided to administer apomorphine (i.v.) thereafter.

5.4.1.2 Effect of apomorphine (i.v.) on ICP rises

Apomorphine (i.v.) dose-dependently increased the frequency of ICP rises per rat and the percentage of rats displaying ICP increases which is consistent with previous reports (Giuliano et al, 2001). ICP increases were initially detected after 0.01 mg/kg and such increases were maximally displayed after 1mg/kg. The higher dose of 5 mg/kg was less effective in eliciting ICP responses than 1mg/kg. Since 0.25 and 1 mg/kg dose of apomorphine produced 5 and 6 mean ICP rises per rat, respectively, we chose the sub-maximal dose of 0.25 mg/kg in all subsequent studies. In contrast to our erectile dose response data, Giuliano and colleagues (2002) administered doses of apomorphine ranging from 0.002-0.25 mg/kg and subsequently used 0.05 mg/kg i.v. dose of apomorphine because this was the lowest dose they found to significantly increase the SUM AUC/BP. We too found 0.05 mg/kg apomorphine to significantly increase ICP responses compared to vehicle; however, we found that such responses were further enhanced after 0.25 and 1mg/kg. Thus, we decided 0.25mg/kg was the most suitable dose of apomorphine for our study because it consistently produced robust increases in ICP and so would allow the oxytocin receptor antagonist (injected in experiments that followed) to act within the efficacious pharmaceutical window. Latency, duration and amplitude of ICP responses after apomorphine injection could not be statistically analysed due to the lack of ICP response in all vehicle treated rats.

Interestingly El-Din and colleagues (2006) found that i.v. injection of apomorphine (0.1-0.2 mg/kg) alone was not sufficient to induce ICP rises. They only found significant changes when apomorphine (i.v.) was administered prior to cavernous nerve stimulation. They believed that the erectogenic effects of apomorphine were only visible when in the presence of some form of sexual stimulation. However, this finding is not consistent with our data or previous studies in conscious and anaesthetised rats, where the pro-erectile effect of apomorphine occurred without any apparent associated sexual stimulation (Giuliano et al, 2001; Hsieh et al, 2004; Martino et al, 2005; Melis et al, 1987, 1989, 2005). So it seems that apomorphine can act as an inducer and facilitator of penile erection suggesting that dopamine acts at multiple sites or it can perhaps activate more than one pro-erectile pathway. We are thus convinced that the powerful erectogenic responses observed in our study are due to the specific inducer actions of apomorphine on central erectile pathways.

El-Din and colleagues (2006) also found 0.1 mg/kg apomorphine was sufficient to potentiate ICP responses to cavernous stimulation, whilst 0.2 mg/kg had no effect when compared to vehicle. They claim these effects were due to the biphasic effects of apomorphine (low doses facilitating penile erection and high doses inhibiting penile erection as reported before (Eaton et al, 1991; Pehek et al, 1988). However, these experimentors only observed the biphasic effects after increasing the dose five- and tenfold and not twofold. Reasons for such discrepancies between our results and those of El-Din and colleagues remain unclear but perhaps the strain of rat used and "n" number per group (both unknown in the study conducted by El-Din and colleagues) were contributing factors. It is likely however, that such contrasts are due to the differential activation of central pathways upon dopamine stimulation alone and in conjunction with other components of the erectile framework.

The hypotensive effect of apomorphine in rats has been reported before (El-Din et al, 2006; Velasco and Luchsinger, 1998 for review). In our study we noticed that lower doses of apomorphine (10 and 150μg/kg) significantly decreased blood pressure, however, the higher doses did not have such a profound hypotensive effect. Rats treated with these two lower doses displayed clear increases in

ICP, thus this slight depressant effect of apomorphine on blood pressure did not seem to have any profound effects on ICP.

5.4.1.3 Effect of an oxytocin receptor antagonist (i.s.) on apomorphine-induced ICP rises

In agreement with other studies (Giuliano et al, 2001; Martino et al, 2005), 30ng of oxytocin given i.s. is able to produce powerful increases in phasic ICP responses. The L4-L6 spinal cord contains the sacral parasympathetic nucleus (SPN) which regulates parasympathetic proerectile outflow. The SPN is partly innervated by oxytocin fibres and possesses oxyocin receptor binding sites (Veronneau-Longueville et al, 1999). Thus, it seems logical to assume that oxytocin action in this area of the lumbar spinal cord is a potent stimulator of spinal proerectile neurons. However, it is important to add however, that the SPN is not comprised exclusively of pro-erectile preganglionic neurons and the SPN is under central control from other neuromodulatory pathways such as serotonin and galanin which have been implicated in erectile function and sexual behaviour (Benelli et al, 1994; Tang et al, 1998). Thus, it would be naive to presume that oxytocinergic transmission in the SPN is the primary spinal inducer of erection. Because of the presence of oxytocinergic constituents in this nucleus it is tempting to hypothesis that expression of penile erection is secondary to local oxytocin effects. However, it is important not to discount the possibility of an indirect effect of oxytocin in the spinal cord or involvement of other known pro-erectile neuromodulators.

Because apomorphine acting in the PVN and oxytocin acting in the L4-L6 spinal cord (due to oxytocin release from the descending paraventriculo-spinal oxytocin pathway) are erectogenic, it seems plausible to suggest that apomorphine elicits penile erection via activation of this descending oxytocin pathway. For the first time we have shown that oxytocin receptor blockade in the lumbosacral spinal cord, reduced the facilitatory effect of apomorphine on ICP rises. This suggests that lumbosacral oxytocin mediates apomorphine-induced penile erection. Both 180ng and 900ng of

the oxytocin receptor antagonist (OXA) significantly reduced apomorphine-induced ICP responses with the 180ng dose being the most effective. It is unclear as to why the 900ng dose of OXA was less effective than the 180ng dose. However, one possible reason may be that 900ng was in fact too high and whilst all the oxytocin receptor binding sites were saturated, the OXA may have acted on other non-specific sites that may act to facilitate penile erection.

Interestingly, the OXA did not completely abolish ICP responses, indicating that spinal oxytocin is not the only neuromediator involved in penile erection and suggests oxytocin has a modulatory rather than a primary role in dopamine-mediated penile erection. The injection site of the OXA lay close to the dorsal horn (DH), dorsal grey commisure (DGC), SPN and intermediolateral grey matter (IML), all of which receive oxytocinergic innervation, which contain oxytocin receptor binding sites and are believed to be involved in sensory and somatic control of erectile pathways (Tang et al, 1998; Veronneau-Longueville et al, 1999). The precise role of each of the above spinal nuclei and involvement of oxytocin, it is not clear. Perhaps direct injection in the SPN may have produced a greater inhibitory effect on apomorphine-induced ICP rises.

In addition to the role of other spinal cord nuclei in the ICP response, the less than profound effect of the OXA on erectile episodes elicited by apomorphine may be partly due to the dose of apomorphine administered. The 0.25 mg/kg dose of apomorphine may have been too high for the OXA to completely attenuate ICP rises. Other studies have used 0.05-0.1 mg/kg as an effective dose to elicit significant increases in ICP, thus perhaps administering a lower dose of apomorphine after OXA injection would have revealed its effects via oxytocin more clearly. This is unlikely to be the case because the lumbosacral spinal cord is the important intergrative site that is believed to generate penile erection. In OXA treated rats, apomorphine did not affect the amplitude, latency or duration of the ICP response suggesting that expression of penile erection is threshold-dependent. Thus, by

increasing the dose of apomorphine, this does not necessarily reflect a concomitant/sequential increase in spinal oxytocin release.

The most likely explanation for the lack of abolishment of apomorphine-induced ICP rises after OXA injection is perhaps due to involvement of other excitatory neuromodulatory pathways in the spinal cord. Descending oxytocin pathways are not the only candidate neurons implicated in spinal control of erectile function. Injection (i.s.) of dopamine, glutamate and melanocortin have all been shown to elicit ICP responses (Giuliano et al, 2001; Rampin et al, 2004; Wessells et al, 2003). Furthermore, it is possible that the paraventricular projection to the lumbosacral spinal cord may not be direct but via the recruitment of serotonergic cells in the nucleus paragigantocellularis. Fibres originating in the PVN have been shown to lie in close apposition to serotonergic cells in the nucleus paragigantocellularis (Bancila et al, 2002). Additionally, intraspinal injection of 5HT 1C and 5HT 2C agonists produces transient increases in ICP (Stafford et al, 2006; Steers et al, 1992) suggesting the existence of a descending excitatory serotonergic pathway.

Finally, OXA treated rats receiving apomorphine did not show any significant depressant effects on blood pressure, thus the reduced facilitatory effect on ICP increases seen in these animals was due to the OXA and not caused by hemodynamic changes in the penile vasculature.

5.4.2. Effect of apomorphine (i.v.) on oxytocin release in the lumbosacral spinal cord

Increases in oxytocin levels have been observed in the PVN, CSF, subarachnoid space and systemic blood during sexual behaviour in rats and humans (Hughes et al, 1987; Kruger et al, 2006; Sansone et al, 2002; Uckert et al, 2003; Waldherr and Neumann, 2007). Dopamine agonists are also known to increase plasma and hippocampal oxytocin concentrations (Melis et al, 1989, 1990). Since we have

shown that blockade of lumbosacral oxytocin receptors reduces the incidence of penile erection stimulated by apomorphine, we investigated whether apomorphine could also increase oxytocin release within the lumbosacral spinal cord of the anaesthetised rat.

Our preliminary data show that oxytocin levels appeared to peak at approximately 60 min after apomorphine (0.25 mg/kg) injection, where we observed a non-significant increase in oxytocin levels in the lumbosacral spinal cord. There were some initial technical difficulties experienced in the experimental set up and an unexpectedly high number of animals did not survive the experimental test period (presumably due to fluid accumulation in the lungs). Thus, the "n" numbers were comparatively low in the control group (n=8 and 6 for apomorphine and vehicle treated groups, respectively) and perhaps partly explain the lack of statistical significance. One surprising finding in this study was that the increase in oxytocin levels was not detected until 60 min after apomorphine injection. This does not fit well with the quick effect of the oxytocin antagonist on apomorphine-induced ICP responses. Nor does it bode well for the generation of penile erection in a sexual behaviour context because the rapid onset of penile erection is required for intromission and ejaculation (which normally occur within 5-10 min during sexual behaviour studies). Furthermore, conscious and anaesthetised animals injected with apomorphine display erections within 15 min (Brioni et al, 2004) and 10 min (see Figure 4.3-4.4), respectively. Thus, one would assume apomorphine-induced oxytocin increases to occur prior to 10 min to elicit penile erection. Interestingly, in a similar study in female rats, the investigators used vaginocervical stimulation to stimulate oxytocin release in the lumbosacral spinal cord. They found an enhancement in oxytocin release approximately 10-15 min after the vaginocervical stimulation (Sansone et al, 2002). Reasons for such discrepancies could be due to the different forms of stimulation of oxyocin release, i.e. mechanical versus pharmacological stimulation and the different pathways recruited by each. It is known that when placed with a receptive female, the male rat will receive olfactory and social cues which facilitate sexual arousal and penile erection and allow for rapid and efficient copulation.

Therefore, it could be said that the increased latency for rises in spinal oxytocin levels in the anaesthetised rat is due to the absence of female oestrus pheromones. This seems unlikely however, as those conscious and anaesthetised males receiving apomorphine in the absence of a female or oestrus cues, achieve penile erection within approximately 10-15 mins. The most likely reason may be that as mentioned previously, the activity of neural pathways can often be less responsive than those in freely-moving rats. Although the central pathways recruited by apomorphine to control penile erection do not seem to be affected by urethane anaesthesia, perhaps those neurons that convey signals to erectile pathways may be augmented.

One other possibility could be that apomorphine stimulates simultaneous and/or sequential release of oxytocin at other central sites during penile erection which may diffuse to target sites that regulate erectile function. We know that apomorphine increases oxytocin levels in the plasma and hippocampus (Melis et al, 1989, 1990). The SON and PVN are two other potential release sites. The pro-erectile melanocortin, α-MSH has been shown to enhance dendritic release of oxytocin within the SON (Sabatier et al, 2003) and copulatory behaviour markedly increases the concentration of oxytocin in the PVN (Waldherr and Neumann, 2007). Thus, it seems tempting to suggest that apomorphine-induced penile erection requires release of oxytocin at multiple central sites implicated in sexual behaviour and not exclusively in the lumbosacral spinal cord.

This latent and marginal increase in spinal oxytocin levels after dopaminergic stimulation further substantiates our previous findings demonstrating that oxytocin action in the lumbosacral spinal cord exhibits a the less than pivotal role in the generation of dopamine-mediated erectile function. Thus, penile erection generated in the conscious male rat in a sexually-exciting environment is more likely due to the summative effect of the activation of various neurotransmitter and neuropeptide pathways as opposed to one neural pathway in particular.

In conclusion, we have shown that the erectogenic ligand, apomorphine, may act centrally to stimulate penile erection partly via the recruitment of paraventricular descending oxytocin neurons and release of oxytocin in the lumbosacral spinal cord. Subsequently, the spinal proerectile neurons (perhaps contained within the SPN) become activated and control peripheral outflow to the penis. It is apparent that penile erection elicited by apomorphine does not exclusively involve this descending oxytocin pathway but rather spinal oxytocin serves a neuromodulatory role in apomorphine-mediate penile erection.

Chapter 6

General discussion

Chapter 6

Chapter 6 General Discussion

It is clear that dopamine has a well established role in the expression of male copulatory behaviour (Hull and Dominguez, 2007 for review). By modulating dopaminergic neurotransmission via the central application of dopamine receptor ligands, expression of penile erection in male rats can be augmented. We have shown that such effects on erectile function appear to be at least partly modulated via the parvocellular and magnocellular oxytocin cells in the hypothalamus of the male rat.

One of the most interesting findings in our studies was the apparent differential involvement of hypothalamic oxytocin cells during dopamine-mediated penile erection. The activation of these neurons appeared to be contextually-specific, with parvocellular oxytocin cells in the PVN being implicated in pharmacologically-induced penile erection and magnocellular oxytocin neurons in the SON mediating physiologically-induced penile erection during copulation (*in-copula* penile erection).

6.1 Parvocellular oxytocin cells and the initiation of penile erection

In our behavioural study, the dopamine receptor agonists Quinelorane (D2/D3) and PD168077 (D4) were highly effective in eliciting penile erection in conscious males. However, Quinelorane but not PD168077 seemed to activate oxytocin neurons in the parvocellular PVN during the expression of penile erection. Interestingly, neither agonist had any effect on Fos expression in oxytocin neurons in the MPN, SON or mPVN.

Currently, the PVN is the only known nucleus to contribute parvocellular oxytocin fibres to the lumbosacral spinal cord (Tang et al, 1998; Veronneau-Longueville et al, 1999; Wagner and Clemens, 1991). From our ICP study in anaesthetised rats, we attempted to block the action of oxytocin released from descending parvocellular oxytocin cells in the lumbosacral spinal cord after systemic delivery of apomorphine. Such disruption of oxytocin action in the lumbosacral spinal cord clearly

impaired the frequency of ICP rises induced by apomorphine. Thus, from our studies in conscious and anaesthetised rats, it seems that oxytocin cells in the parvocellular PVN are one potential pathway recruited by dopamine receptor agonists during the expression of penile erection. The dopamine receptor(s) believed to subserve the pharmacologically-induced penile erection we observed in our studies remains unknown. Apomorphine acts on all dopamine receptors, however, Quinelorane preferentially acts on D2 and D3 receptors and PD168077 is a selective D4 receptor agonist. Since Quinelorane but not PD168077 selectively activated the parvocellular PVN during the expression of penile erection, it is tempting to suggest that activation of the dopamine-oxytocin pathway in eliciting penile erection relies upon stimulation of D2 and/or D3 receptors. Moreover, this idea is reinforced by the finding that parvocellular oxytocin cells in the PVN express D2 and D3 receptors. Dopamine D4 receptor activation was also effective in eliciting penile erection. However, in contrast, the D4 agonist had no effect on hypothalamic oxytocin neurons and we found D4 receptors to be predominantly expressed by magnocellular but not parvocellular oxytocin neurons. So perhaps D4 receptor-mediated penile erection operates via alternative neural circuitries (such as GABA as described previously) or an as yet unknown pathway.

Some researchers believe that parvocellular oxytocin cells have at most a minimal role in penile erection. This is partly due to the fact that these cells exist in lower numbers and release comparatively lower levels of oxytocin when compared to the robust magnocellular oxytocin system. As previously mentioned, lesioning of the descending oxytocin pathway selectively impairs penile erection parameters, particularly oxytocin- and apomorphine-induced erectile events but rats show no obvious deficits in copulatory function (Ackerman et al, 1997; Argiolas et al, 1987; Liu et al, 1997). Taken together, the findings from our studies and those of others seem to suggest that parvocellular oxytocin pathways originating in the PVN serve as important neural substrates in the initiation and achievement of penile erection. However, a possible role for parvocellular oxytocin cells in those sexually-associated events occurring after penile erection have yet to be established.

To further understand the exact central mechanism(s) whereby dopamine exerts its pro-erectile effects, it is important to mention that the spinal generator of erectile events is under tonic supraspinal

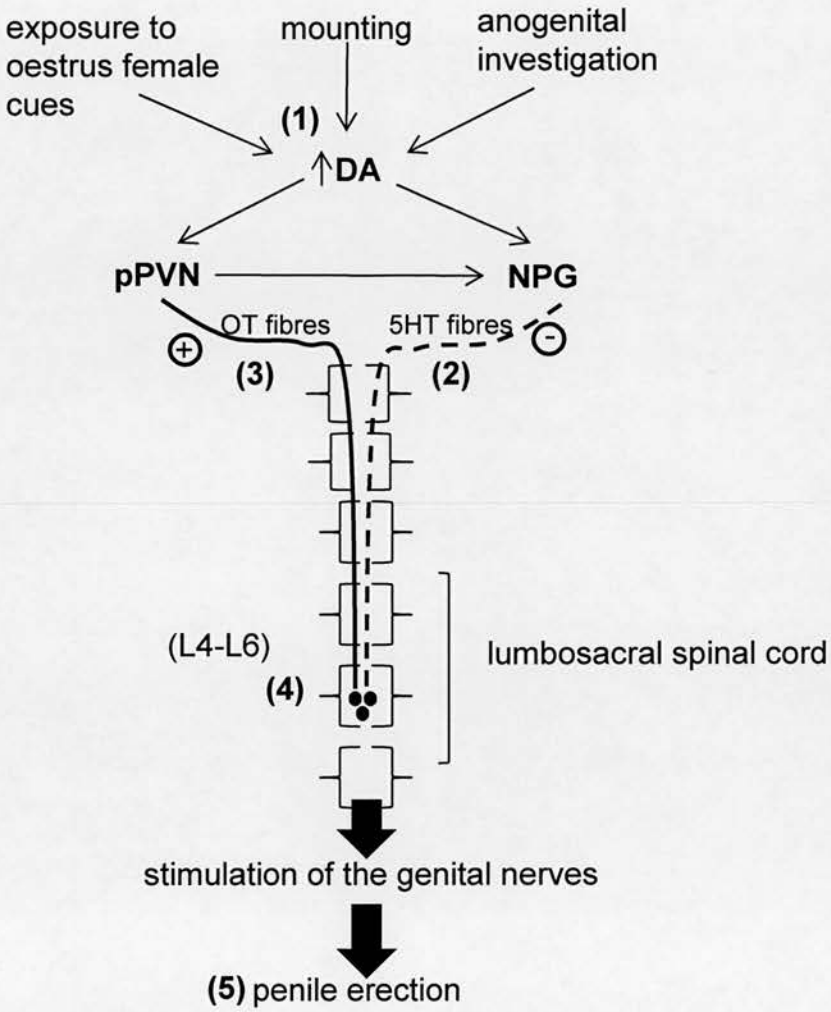
inhibitory control via descending serotonergic fibres located in the nucleus paragigantocellularis (NPG). Thus, dopamine acting on descending oxytocin fibres alone is probably not sufficient to stimulate penile erection. We know that the PVN sends direct projections to the NPG (Bancila et al, 2002) and serotonergic fibres in the NPG have been implicated in the inhibition of penile reflexes (Marson et al, 1992; Marson and Mckenna, 1992). So, it may be that dopamine acts in a dual manner to disinhibit the inhibitory spinal serotonergic influence whilst activating the descending excitatory oxytocin pathway. Once parasympathetic neurons in the SPN have been sufficiently depolarised by oxytocin acting in the lumbosacral spinal, there is subsequent stimulation of pelvic and cavernosal nerves to elicit penile erection (see Figure 6.1).

6.2 Magnocellular oxytocin cells and intromission

We have shown that penile erection elicited by central and systemic delivery of dopamine agonists partly involves stimulation of parvocellular oxytocin neurons. Conversely, when examining the effect of D2-like receptor antagonists on physiologically-induced penile erection during sexual activity, no significant effects on parvocellular oxytocin neurons were observed. We have seen that blockade of D3 receptors partially inhibits penile erection via a non-oxytocinergic pathway in the MPN, SON and PVN (as identified by the Fos only data). However, it was surprising to find that by inhibiting the actions of endogenous dopamine at the D4 receptors, we effectively reduced the expression of penile erection partly via an oxytocinergic pathway in the SON but not in the MPN or PVN. This suggests a role for magnocellular but not parvocellular oxytocin cells during *in-copula* penile erection.

Such differential involvement of parvocellular and magnocellular oxytocin cells in the expression of penile erection provides some new insight into those neural processes underlying the initiation and maintenance of penile rigidity. It is apparent that pharmacologically- and physiologically-induced penile erections seem to recruit different pathways in the generation of penile erection. Both stimuli appear to operate via distinct neuronal populations, and in some cases sub-populations, within the MPN, SON and PVN. From these behavioural studies, it is apparent that oxytocin neurons in the

Figure 6.1: Involvement of parvocellular oxytocin cells in the initiation of penile erection

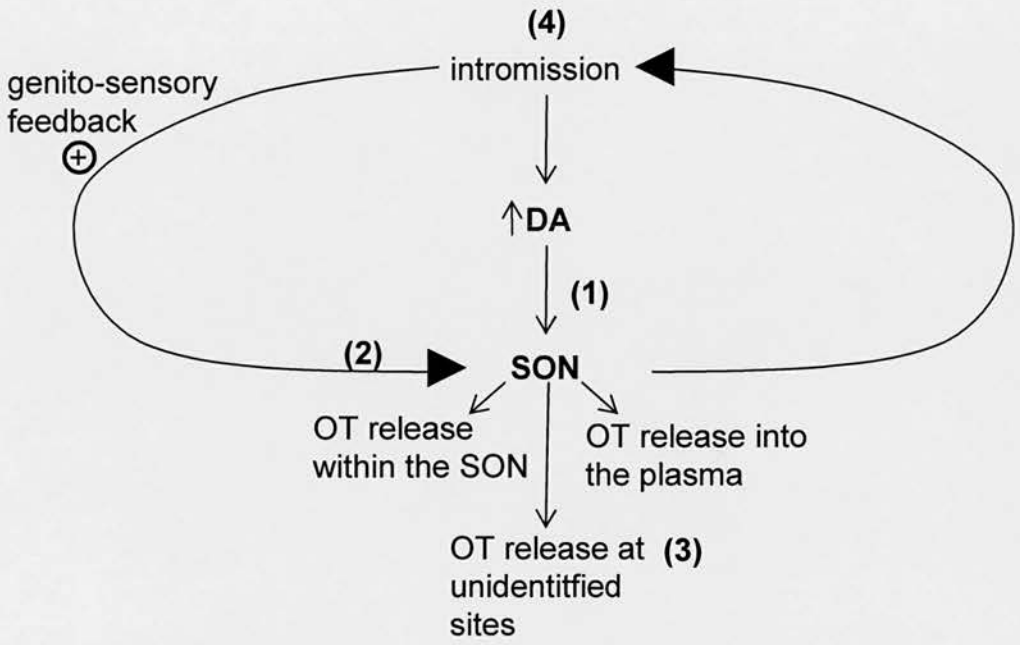


Role of parovcellular oxytocin cells in the generation of penile erection. Those sexually-relevant stimuli known to elicit penile erection increase incertohypothalamic-derived dopamine concentrations (1), dopamine then acts either directly and/or indirectly to disinhibit descending serotonergic control (2) and to excite descending parovcellular paraventriculospinal oxytocin cells (3), oxytocin released in the L4-L6 spinal cord activates preganglionic parasympathetic neurons in the sacral parasympathetic nucleus (4), which stimulates peripheral output to the penis and elicits penile erection (5). DA=dopamine, 5HT-serotonin, OT=oxytocin, pPVN=parvocellular PVN, NPG=nucleus paragigantocellularis.

parvocellular PVN mediate penile erection induced pharmacologically. However, blockade of dopamine D4 receptors during physiologically-induced penile erection (as experienced during copulation) appears to affect only the magnocellular oxytocin neurons in the SON (but not in magnocellular PVN). Could it be that non-contact penile erection induced by dopamine receptor agonists (or sexual stimuli which promote dopamine release such as exposure to remote female oestrus cues), involves parvocellular PVN oxytocin neurons; however, *in-copula* penile erection selectively activates magnocellular oxytocin neurons? The mechanism by which dopamine excites magnocellular oxytocin cells has yet to be explored. However, it could be postulated that during intromission, dopamine levels increase in the SON and such elevated dopaminergic levels coupled with sensory feedback from the penis act to disinhibit the GABAergic influence on oxytocin cells and so facilitate oxytocinergic activation. Paraventricular and supraoptic oxytocin cells in particular are known to be activated upon tactile manipulation of the glans penis and stimulation of the dorsal penile nerve (Honda et al, 1999; Yanagimoto et al, 1996). This suggests that genito-sensory signals transmitted by the dorsal penile nerve, selectively activate magnocellular oxytocin cells in the PVN and SON. Thus, it seems that magnocellular oxytocin activation requires penile erection *and* genito-sensory input as experienced during intromission in copulating rats (see Figure 6.2). Furthermore, a more prominent role for magnocellular oxytocin neurons during the consummatory elements of sexual behaviour as opposed to non-contact erections was further substantiated when it was shown that significant disruptions in copulatory function were only observed after lesioning of both the parvocellular and magnocellular PVN (Ackerman et al, 1997).

It is interesting to point out that while supraoptic magnocellular oxytocin cellular activation has been implicated in the regulation of *in-copula* penile erection, no such effect was observed in magnocellular PVN oxytocin cells. This was an unexpected finding as a similar homogenous population of magnocellular oxytocin cells are thought to exist in both the SON and PVN and both have been shown to be activated during intromission (Caquineau et al, 2006). Perhaps by disrupting normal dopaminergic neurotransmission (as we have by administering dopamine antagonists), this has more of an impact on the functioning of oxytocin cells in the SON during sexual behaviour as

Figure 6.2: Involvement of magnocellular oxytocin cells during intromission



Role of supraoptic magnocellular oxytocin cells during intromission in the copulating rat. During intromission there is an increase in incertohypothalamo-derived dopamine concentrations (1) as well as peripheral sensory feedback from the genitals (2) which inturn stimulate magnocellular oxytocin release within the SON, plasma and at other unknown sites (3) to facilitate intromisssion (4). DA=dopamine, OT=oxytocin, SON=supraoptic nucleus.

opposed to those in the magnocellular PVN which may have more of a modulatory role. We know that dopamine agonists and antagonists can act locally in the SON to inhibit or facilitate GABAergic neurotransmission, respectively and so subsequently enhance or decrease oxytocin release, respectively (Azdad et al, 2003). However, this is not known for paraventricular magnocellular oxytocin cells, so it may be that those neural inputs impinging on oxytocin cells in the mPVN may differentially modulate oxytocinergic transmission compared to those in the SON, although this is highly speculative.

In addition to the involvement of magnocellular oxytocin neurons in erectile function, it is important to draw attention to the potential role of magnocellular vasopressin cells in the SON (and perhaps the PVN) in mediating the pro-erectile effects of dopamine. In our studies, we have shown that blockade of D3 receptors in the SON and PVN attenuates neuronal activation of non-oxytocinergic cells during *in-copula* penile erection (Chapter 3). Because the SON and PVN contain large populations of oxytocin and vasopressin cells, it is quite possible that the neuronal phenotype expressing Fos may include vasopressin. Furthermore, we also demonstrated an abundant expression of D2-like receptors on non-oxytocinergic neuronal populations in the SON and PVN (Chapter 4) which are also likely to be vasopressin in origin. So, this introduces an exciting prospect whereby a dopamine-vasopressin link exists in addition to a dopamine-oxytocin pathway in mediating erectile function. Of the very few studies investigating the role of vasopressin and male sexual behaviour, it has been shown that microinjection of vasopressin into the PVN or hippocampus stimulates penile erection; however, it was less erectogenic than oxytocin (Argiolas et al, 1986; Argiolas et al, 1989; Melis et al, 1986). In addition, paraventriculospinal vasopressin fibres appear to have some excitatory influence over the activity of penile striated muscles (Ogier et al, 2006) which are important for penile rigidity and facilitating ejaculation. To date there are no published studies demonstrating an interaction between dopamine and vasopressin in a sexual context. We know that central or intra-SON injection of dopamine stimulates vasopressin release *in vitro* and *in vivo* (Bridges et al, 1975; Urano and Kobayashi, 1978). Behavioural studies have also shown an activation of mesolimbic dopamine pathways and vasopressin systems during conditioned partner preference and pair bonding associated

with aggression (Smeltzer et al, 2006; Gobrogge et al, 2007; Lim et al, 2004). However, the implication of a dopamine-vasopressin relationship subserving aspects of masculine sexual behaviour makes for an exciting area of neurobiology yet to be explored.

6.3 Summary

Thus, it seems that two functional oxytocinergic groups may exist in the male rat to facilitate penile erection. Firstly, a group of "low threshold" parvocellular oxytocin cells which can be stimulated after endogenous release of dopamine in the presence of a sexually-relevant stimulus such as exposure to female oestrus cues and/or mounting behaviour. Such interactions between dopamine and parvocellular PVN oxytocin neurons are required for the initiation phase of penile erection but not for those sexually-associated events occurring after achievement of penile erection (eg. intromission and pelvic thrusting). As we have shown, penile erection alone is not sufficient to stimulate magnocellular oxytocin cells. Thus, a second group of "high threshold" magnocellular oxytocin neurons may exist which are activated by endogenous dopamine during intromission when there is sensory feedback from the genitals. Perhaps the combined actions of endogenous dopamine and peripheral feedback are necessary to activate magnocellular oxytocin cells. It would seem then that such magnocellular oxytocin involvement may serve as an important neural basis regulating those events occurring after achievement of penile erection, such as intromission, pelvic thrusting and even ejaculation. The above explanation is hypothetical and does not take into account other potential neural pathways that can indirectly and directly influence oxytocinergic transmission in the brain or that oxytocin may be released sequentially or concurrently at multiple release sites in the CNS during penile erection. It is likely that a delicate and co-ordinated oxytocinergic system exists whereby there is release of oxytocin within the SON, PVN, plasma and the spinal cord which governs central and peripheral output and culminates in penile erection.

Finally, it is very apparent that the central regulation of penile erection is not under the control of any one brain nucleus in particular and so it would be naive to consider the MPN, SON and PVN to be the

only major brain regions responsible for initiating penile erection. There appears to be reciprocal connections between the nucleus accumbens, amygdala, striatum, hippocampus and the hypothalamus to name but a few, all of which have been implicated in co-ordinating male sexual responses (Kippin et al, 2004; Temel et al, 2006 for review). Thus, we know that a very complex and intricate system exists which integrates peripheral and olfactory stimulatory cues with brain pathways that modulate the spinal pro-erectile network. In our studies we have only looked at one particular hypothalamic pathway (dopamine-oxytocin) and one specific sexual element, penile erection. The role of dopaminergic-oxytocinergic crosstalk in those events occurring prior to (sexual motivation) and immediately after penile erection (maintenance of penile rigidity required for intromission) remain unknown. However, further investigation of the neurochemical correlates responsible for the appetitive and consummatory aspects of male sexual behaviour would help provide new insight into our rather blurred current interpretation. Finally, it is necessary to better understand the role of those nuclei that provide a range of neural inputs into the main pro-erectile brain centres and also the complex intra-hypothalamic neural circuitries that exist between the MPN, SON and PVN which are largely unknown.

Chapter 7

General conclusion

Chapter 7

Chapter 7 General Conclusion

Although some may view impaired erectile function as a natural age-related progressive decline in sexual functioning, the increasing prevalence of erectile dysfunction among middle-aged men and its physical and psychological impact on sufferer's lives make this condition an important area of neurophysiology yet to be fully explored.

Elucidating those central and peripheral pathways responsible for generating penile erection has proved complex because the exact causes of impaired penile erection remain ambiguous. Vascular, neurological and hormonal influences are all believed to be causative. However, in addition to this, a range of underlying vascular diseases such as diabetes, hypertension and atherosclerosis, all of which can disrupt blood flow to the penile vasculature, can pre-dispose individuals to impaired erectile function. Additionally, psychogenic factors can also have a marked effect on penile erection as inferred by studies showing men suffering from depressive-like illnesses exhibit a higher incidence of erectile dysfunction (Greiner and Weigel, 1996 for review). Thus, successful treatment of this condition has proved difficult because targeting those central and peripheral processes that are disrupted during erectile dysfunction may not be sufficient to treat penile erection in some individuals where perhaps a more holistic approach may be more beneficial. However, it is important to stress that in most cases of erectile dysfunction the causes are organic. Although a curative therapy is not available for this form of male sexual dysfunction, there has been some commercial success with a few select pharmacological agents that are designed to target different stages of the erectile pathway in men.

7.1 Modern treatment for erectile dysfunction

Modern pharmacological treatments for erectile dysfunction have included sublingual delivery of apomorphine and oral administration of phosphodiesterase-5 (PDE5) inhibitors (Dinsmore, 2004).

Apomorphine as stated earlier is a potent inducer and facilitator of penile erection and has proven to be relatively effective in men with mild to moderate erectile impairments (Altwein and Keuler, 2001; Briganti et al, 2004 for review). However, it is generally short-lasting and can produce severe side effects including nausea and emesis. Due to its strong inducer and facilitatory effects, there have been some reports of priapism (prolonged painful erections) in men suffering from multiple sclerosis and Parkinson's disease (Landtblom, 2006; O'Sullivan and Hughes, 1998). Because these side effects can be too severe to provide any long term treatment, development of alternative pharmacological therapies are needed that are more efficacious and possess a larger therapeutic window. Interestingly, very recently intranasal delivery of apomorphine in rats was shown to have improved efficacy with fewer side effects (Lu et al, 2008). Thus, an intranasal route of administration of apomorphine may be a potentially effective alternative for treatment in humans and aid in combating the percentage of patients experiencing adverse side effects associated with dopamine-enhancing drugs.

Phosphodiesterase (PDE) inhibitors, particularly PDE5 inhibitors are another form of pharmacological treatment for erectile dysfunction which is currently the most popular of oral therapies (Miner and Seftel, 2007; Sperling et al, 2003). PDE5 inhibitors act peripherally on penile tissue to prevent the breakdown of NO-stimulated cGMP levels. Due to the high concentration of cGMP in penile tissue, there is a subsequent decrease in intracellular calcium levels which potentiates the relaxation of penile smooth muscle and so facilitates penile erection (Corbin et al, 2002). PDE5 inhibitors have provided safe, effective treatment in some men, causing only minor side effects (Dinsmore, 2004) and there is a low risk of priapism (Saenz et al, 2001). However, because NO release precedes cGMP formulation, PDE5 inhibitors are only effective in stimulating penile erection following sexual arousal. Thus, PDE5 inhibitors aid in maintaining penile rigidity but unlike apomorphine, they have no effect on the initiation of penile erection. They acts as facilitators and are not inducers of penile erections. Thus some men may not respond to or display reduced responsiveness to these peripheral agents.

So, it can be seen that recent advancements in erectile dysfunction therapies have proved to be relatively successful in some patients; however, due to the relative non-specificity of apomorphine and the purely facilitatory effect of PDE5 inhibitors, it is essential to develop other therapeutic alternatives that will be equally efficacious in inducing and maintaining penile erection without severely impacting on normal physiological functions.

7.2 Central oyxtocin sites as potential therapeutic targets

In this thesis, we have tried to establish a link between central dopamine and oxytocin pathways with the aim of elucidating one potential neuroanatomical pathway that may mediate erectile function. From our findings and other published data (Argiolas and Melis, 2004 for review), we are convinced that endogenous dopamine may act (directly/indirectly) via oxytocin in the CNS to induce penile erection. Due to their large oxytocinergic populations, the PVN and SON may serve as two potential integration sites. Such activation of oxytocin neurons in these nuclei can result in widespread oxytocin release in the CNS and in the plasma resulting in the activation of pro-erectile pathways. Oxytocin cells are not solely responsive to dopamine agonists but are also believed to respond to a range of erectogenic agents including excitatory amino acids, oxytocin, NO and hexarelin analogues (Melis and Argiolas, 2003 for review) suggesting that hypothalamic oxytocin pathways are a key component of penile erection. Thus, this gives rise to an area of sexual pharmacology yet to be explored.

Oxytocin has become a key clinical target due to its potent erectogenic effects in pre-clinical studies (Argiolas and Melis, 2004 for review; Kita et al, 2006; Martino et al, 2005) and seemingly lack of severe side effects to patients when given in low doses (Smith and Merrill, 2006). However, to date there are no oxytocin agonists clinically available for the treatment of erectile dysfunction. In modern obstetrics, oxytocics have a clearly established role (den Hertog et al, 2001). Clinical management of pre-term labour and post partum haemorrhaging, both of which are influenced by oxytocinergic inputs and are due to enhanced oxytocinergic neurotransmission; are effectively managed by systemic

delivery of oxytocin antagonists. Similarly, oxytocin agonists can be administered to increase uterine activity and so induce labour in women who have reached full-term in their pregnancy (Romero et al, 2000; Shyken and Petrie, 1995a, 1995b; Smith and Merrill, 2006). Other clinical and pre-clinical studies have revealed that systemic delivery of oxytocin antagonists are able to block uterine contractions stimulated by oxytocin in women, monkey and rats (Croci et al, 2007; Pettibone et al, 1995; Serradeil-Le Gal et al, 2004) and delay parturition in pregnant rats and monkeys (Croci et al, 2007; Serradeil-Le Gal et al, 2004). Taken together, these findings show that manipulation of oxytocin levels via the use of oxytocin ligands can be extremely effective in facilitating child birth and treating post partum bleeding in women. This strongly suggests that pharmacological agonists of oxytocin may also have some clinical relevance regarding the treatment of erectile impairment in men. Thus, targeting central oxytocin pathways may serve as one potential way of improving treatment of sexual dysfunction in men.

It is too premature to speculate on the potential therapeutic value of combination therapy involving dopaminergic and oxytocingeric stimulants. However, this may be one area of pharmacotherapeutic development that would benefit from more exploratory and investigative research and aid in the continued development of safe and effective treatment for erectile dysfunction.

Ackerman,A.E., Lange,G.M. & Clemens,L.G. (1997) Effects of paraventricular lesions on sex behavior and seminal emission in male rats. *Physiology & Behavior*, 63, 49-53.

Adachi,H., Sato,Y., Kato,R., Hisasue,S., Suzuki,K., Masumori,N., Itoh,N. & Tsukamoto,T. (2003) Direct evidence of facilitative actions of dopamine in the medial preoptic area on reflexive and noncontact erections in male rats. *Journal of Urology*, 169, 386-389.

Adan,R.A. & Gispen,W.H. (1997) Brain melanocortin receptors: from cloning to function. *Peptides*, 18, 1279-1287.

Agmo,A., Choleris,E., Kavaliers,M., Pfaff,D.W. & Ogawa,S. (2008) Social and sexual incentive properties of estrogen receptor alpha, estrogen receptor beta, or oxytocin knockout mice. *Genes Brain Behav.* 7(1):70-7.

Allard,J., Bernabe,J., Derdinger,F., Alexandre,L., McKenna,K. & Giuliano,F. (2002) Selegiline enhances erectile activity induced by dopamine injection in the paraventricular nucleus of the hypothalamus in anesthetized rats. *International Journal of Impotence Research*, 14, 518-522.

Allard, J., Truitt, WA., McKenna, KE., Coolen LM. (2005) Spinal cord control of ejaculation. *World J Urol.*, 23(2):119-26.

Altwein,J.E. & Keuler,F.U. (2001) Oral treatment of erectile dysfunction with apomorphine SL. *Urol.Int.*, 67, 257-263.

Amico,J.A., Layden,L.M., Pomerantz,S.M. & Cameron,J.L. (1993) Oxytocin and vasopressin secretion in monkeys administered apomorphine and a D2 receptor agonist. *Life Sci.*, 52, 1301-1309.

Andersson,K.E. (2000) Neurotransmitters: central and peripheral mechanisms. *Int.J.Impot.Res.*, 12 Suppl 4, S26-S33.

Antonelli,T., Fuxe,K., Agnati,L., Mazzoni,E., Tanganelli,S., Tomasini,M.C. & Ferraro,L. (2006) Experimental studies and theoretical aspects on A2A/D2 receptor interactions in a model of Parkinson's disease. Relevance for L-dopa induced dyskinesias. *J.Neurol.Sci.*, 248, 16-22.

Aragona,B.J., Liu,Y., Yu,Y.J., Curtis,J.T., Detwiler,J.M., Insel,T.R. & Wang,Z. (2006) Nucleus accumbens dopamine differentially mediates the formation and maintenance of monogamous pair bonds. *Nat Neurosci.*, 9(1):133-9.

Arendash,G.W. & Gorski,R.A. (1983) Effects of Discrete Lesions of the Sexually Dimorphic Nucleus of the Preoptic Area Or Other Medial Preoptic Regions on the Sexual-Behavior of Male-Rats. *Brain Research Bulletin*, 10, 147-154.

Argiolas,A., Melis,M.R. & Gessa,G.L. (1985) Intraventricular Oxytocin Induces Yawning and Penile Erection in Rats. *European Journal of Pharmacology*, 117, 395-396.

Argiolas,A., Melis,M.R. & Gessa,G.L. (1986) Oxytocin - An Extremely Potent Inducer of Penile Erection and Yawning in Male-Rats. *European Journal of Pharmacology*, 130, 265-272.

Argiolas,A., Melis,M.R., Mauri,A. & Gessa,G.L. (1987) Paraventricular Nucleus Lesion Prevents Yawning and Penile Erection Induced by Apomorphine and Oxytocin But Not by Acth in Rats. *Brain Research*, 421, 349-352.

Argiolas,A., Melis,M.R., Vargiu,L. & Gessa,G.L. (1987) d(CH2)5Tyr(Me)-[Orn8]vasotocin, a potent oxytocin antagonist, antagonizes penile erection and yawning induced by oxytocin and apomorphine, but not by ACTH-(1-24). *Eur.J.Pharmacol.*, 134, 221-224.

Argiolas,A., Collu,M., D'Aquila,P., Gessa,G.L., Melis,M.R. & Serra,G. (1989) Apomorphine stimulation of male copulatory behavior is prevented by the oxytocin antagonist d(CH2)5 Tyr(Me)-Orn8-vasotocin in rats. *Pharmacol.Biochem.Behav.*, 33, 81-83.

Argiolas,A., Melis,M.R., Stancampiano,R. & Gessa,G.L. (1990) Omega-conotoxin prevents apomorphine- and oxytocin-induced penile erection and yawning in male rats. *Pharmacol.Biochem.Behav.*, 37, 253-257.

Argiolas,A. & Melis,M.R. (1995) Neuromodulation of penile erection: An overview of the role of neurotransmitters and neuropeptides. *Progress in Neurobiology*, 47, 235-255.

Argiolas,A. & Melis,M.R. (1995) Oxytocin-induced penile erection. Role of nitric oxide. *Adv.Exp.Med.Biol.*, 395, 247-254.

Argiolas,A. (1999) Neuropeptides and sexual behaviour. *Neuroscience and Biobehavioral Reviews*, 23, 1127-1142.

Argiolas,A. & Melis,M.R. (2004) The role of oxytocin and the paraventricular nucleus in the sexual behaviour of male mammals. *Physiology & Behavior*, 83, 309-317.

Argiolas,A. & Melis,M.R. (2005) Central control of penile erection: Role of the paraventricular nucleus of the hypothalamus. *Progress in Neurobiology*, 76, 1-21.

Ariano,M.A., Monsma,F.J., Jr., Barton,A.C., Kang,H.C., Haugland,R.P. & Sibley,D.R. (1989) Direct visualization and cellular localization of D1 and D2 dopamine receptors in rat forebrain by use of fluorescent ligands. *Proc.Natl.Acad.Sci.U.S.A*, 86, 8570-8574.

Arletti,R., Bazzani,C., Castelli,M. & Bertolini,A. (1985) Oxytocin Improves Male Copulatory Performance in Rats. *Hormones and Behavior*, 19, 14-20.

Armagan,A., Kim,N.N., Goldstein,I. & Traish,A.M. (2006) Dose-response relationship between testosterone and erectile function: evidence for the existence of a critical threshold. *J.Androl*, 27, 517-526.

assi-Benelli,A., Ferrari,F. & Quarantotti,B.P. (1979) Penile erection induced by apomorphine and N-n-propyl-norapomorphine in rats. *Arch.Int.Pharmacodyn.Ther.*, 242, 241-247.

Azdad,K., Piet,R., Poulain,D.A. & Oliet,S.H. (2003) Dopamine D4 receptor-mediated presynaptic inhibition of GABAergic transmission in the rat supraoptic nucleus. *J.Neurophysiol.*, 90, 559-565.

Badoer E. (2001) Hypothalamic paraventricular nucleus and cardiovascular regulation. *Clin Exp Pharmacol Physiol.* 28(1-2):95-9.

Bagdy,G., Kalogeras,K.T. & Szemeredi,K. (1992) Effect of 5-HT1C and 5-HT2 receptor stimulation on excessive grooming, penile erection and plasma oxytocin concentrations. *Eur.J.Pharmacol.*, 229, 9-14.

Bagdy,G. & Kalogeras,K.T. (1993) Stimulation of 5-HT1A and 5-HT2/5-HT1C receptors induce oxytocin release in the male rat. *Brain Res.*, 611, 330-332.

Bagdy,G. & Makara,G.B. (1994) Hypothalamic paraventricular nucleus lesions differentially affect serotonin-1A (5-HT1A) and 5-HT2 receptor agonist-induced oxytocin, prolactin, and corticosterone responses. *Endocrinology*, 134, 1127-1131.

Bagdy,G. & Makara,G.B. (1995) Paraventricular nucleus controls 5-HT2C receptor-mediated corticosterone and prolactin but not oxytocin and penile erection responses. *Eur.J.Pharmacol.*, 275, 301-305.

Bagdy,G. (1996) Role of the hypothalamic paraventricular nucleus in 5-HT1A, 5-HT2A and 5-HT2C receptor-mediated oxytocin, prolactin and ACTH/corticosterone responses. *Behav.Brain Res.*, 73, 277-280.

Bale,T.L., Davis,A.M., Auger,A.P., Dorsa,D.M. & McCarthy,M.M. (2001) CNS region-specific oxytocin receptor expression: importance in regulation of anxiety and sex behavior. *J Neurosci.* 21(7):2546-52.

Balfour,M.E., Yu,L. & Coolen,L.M. (2004) Sexual behavior and sex-associated environmental cues activate the mesolimbic system in male rats. *Neuropsychopharmacology.*, 29(4):718-30.

Balthazart,J. & Ball,G.F. (2007) Topography in the preoptic region: differential regulation of appetitive and consummatory male sexual behaviors. *Front Neuroendocrinol.*, 28, 161-178.

Bancila,M., Verge,D., Rampin,O., Backstrom,J.R., Sanders-Bush,E., McKenna,K.E., Marson,L., Calas,A. & Giuliano,F. (1999) 5-Hydroxytryptamine2C receptors on spinal neurons controlling penile erection in the rat. *Neuroscience*, 92, 1523-1537.

Bancila,M., Giuliano,F., Rampin,O., Mailly,P., Brisorgueil,M.J., Calas,A. & Verge,D. (2002) Evidence for a direct projection from the paraventricular nucleus of the hypothalamus to putative serotoninergic neurons of the nucleus paragigantocellularis involved in the control of erection in rats. *European Journal of Neuroscience*, 16, 1240-1248.

Bancroft,J. (2005) The endocrinology of sexual arousal. *J.Endocrinol.*, 186, 411-427.

Baron,R. & Janig,W. (1991) Afferent and sympathetic neurons projecting into lumbar visceral nerves of the male rat. *J.Comp Neurol.*, 314, 429-436.

Basu,A. & Ryder,R.E.J. (2004) New treatment options for erectile dysfunction in patients with diabetes mellitus. *Drugs*, 64, 2667-2688.

Baum,M.J. (2003) Activational and organizational effects of estradiol on male behavioural neuroendocrine function. *Scand J Psychol.* 44(3):213-20.

Bazzett,T.J., Eaton,R.C., Thompson,J.T., Markowski,V.P., Lumley,L.A. & Hull,E.M. (1991) Dose Dependent D2 Effects on Genital Reflexes After Mpoa Injections of Quinelorane and Apomorphine. *Life Sciences*, 48, 2309-2315.

Beck,J., Bialy,M. & Kostowski,W. (2002) Effects of D(1) receptor agonist SKF 38393 on male rat sexual behavior and postcopulatory departure in the goal compartment-runway paradigm. *Physiol Behav.*, 76, 91-97.

Becker,P.L. (1996) Quantitative fluorescence measurements. *In Fluorescence Imaging Spectroscopy and Microscopy*; Wang,X.F., Herman,B.; Eds John Wiley and Sons, Inc. New York, 1-29.

Beckett,S.D., Walker,D.F., Hudson,R.S., Reynolds,T.M. & Purohit,R.C. (1975a) Corpus spongiosum penis pressure and penile muscle activity in the stallion during coitus. *Am J Vet Res.* 36(4 Pt.1):431-3.

Beckett,S.D., Purohit,R.C. & Reynolds,T.M. (1975b) The corpus spongiosum penis pressure and external penile muscle activity in the goat during coitus. *Biol Reprod.* 12(2):289-92.

Ben,Z., I, Tessler,R., Cohen,L., Lerer,E., Raz,Y., Bachner-Melman,R., Gritsenko,I., Nemanov,L., Zohar,A.H., Belmaker,R.H., Benjamin,J. & Ebstein,R.P. (2006) Polymorphisms in the dopamine D4 receptor gene (DRD4) contribute to individual differences in human sexual behavior: desire, arousal and sexual function. *Mol.Psychiatry*, 11, 782-786.

Benelli,A., Arletti,R., Bertolini,A., Menozzi,B., Basaglia,R. & Poggioli,R. (1994) Galantide stimulates sexual behaviour in male rats. *Eur.J.Pharmacol.*, 260, 279-282.

Benoussaidh,A., Maurin,Y. & Rampin,O. (2005) Possible neural mediation of the central effects of oxytocin on uterine motility. *Am.J.Physiol Regul.Integr.Comp Physiol*, 289, R798-R804.

Bernabe,J., Rampin,O., Giuliano,F. & Benoit,G. (1995) Intracavernous pressure changes during reflexive penile erections in the rat. *Physiol Behav.*, 57, 837-841.

Bernabe,J., Rampin,O., Sachs,B.D. & Giuliano,F. (1999) Intracavernous pressure during erection in rats: an integrative approach based on telemetric recording. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, 276, R441-R449.

Bertolini,A. & Gessa,G.L. (1981) Behavioral effects of ACTH and MSH peptides. *J.Endocrinol.Invest*, 4, 241-251.

Bialy,M. & Kaczmarek,L. (1996) c-Fos expression as a tool to search for the neurobiological base of the sexual behaviour of males. *Acta Neurobiol.Exp.(Wars.)*, 56, 567-577.

Bialy,M. & Sachs,B.D. (2002) Androgen implants in medial amygdala briefly maintain noncontact erection in castrated male rats. *Horm Behav.* 42(3):345-55.

Bingham,B., Williamson,M. & Viau,V. (2006) Androgen and estrogen receptor-beta distribution within spinal-projecting and neurosecretory neurons in the paraventricular nucleus of the male rat. *J Comp Neurol.* 499(6):911-23.

Bitner,R.S., Nikkel,A.L., Otte,S., Martino,B., Barlow,E.H., Bhatia,P., Stewart,A.O., Brioni,J.D., Decker,M.W. & Moreland,R.B. (2006) Dopamine D4 receptor signaling in the rat paraventricular hypothalamic nucleus: Evidence of natural coupling involving immediate early gene induction and mitogen activated protein kinase phosphorylation. *Neuropharmacology*, 50, 521-531.

Bitran,D., Thompson,J.T., Hull,E.M. & Sachs,B.D. (1989) Quinelorane (LY163502), a D2 dopamine receptor agonist, facilitates seminal emission, but inhibits penile erection in the rat. *Pharmacol.Biochem.Behav.*, 34, 453-458.

Bjorklund,A., Lindvall,O. & Nobin,A. (1975) Evidence of an incerto-hypothalamic dopamine neurone system in the rat. *Brain Res.*, 89, 29-42.

Bloch,G.J., Eckersell,C. & Mills,R. (1993) Distribution of galanin-immunoreactive cells within sexually dimorphic components of the medial preoptic area of the male and female rat. *Brain Res.*, 620, 259-268.

Bloch,.G.J., Butler,P.C. & Kohlert,J.G. (1996) Galanin microinjected into the medial preoptic nucleus facilitates female- and male-typical sexual behaviors in the female rat. *Physiol Behav.* 59(6):1147-54.

Bloch,G.J., Butler,P.C., Eckersell,C.B. & Mills,R.H. (1998) Gonadal steroid-dependent GAL-IR cells within the medial preoptic nucleus (MPN) and the stimulatory effects of GAL within the MPN on sexual behaviors. *Ann.N.Y.Acad.Sci.*, 863, 188-205.

Bosch ,O.J., Krömer,S.A., Brunton,P.J. & Neumann,I.D. (2004) Release of oxytocin in the hypothalamic paraventricular nucleus, but not central amygdala or lateral septum in lactating residents and virgin intruders during maternal defence. *Neuroscience.* 124(2):439-48.

Bouthenet,M.L., Souil,E., Martres,M.P., Sokoloff,P., Giros,B. & Schwartz,J.C. (1991) Localization of dopamine D3 receptor mRNA in the rat brain using in situ hybridization histochemistry: comparison with dopamine D2 receptor mRNA. *Brain Res.*, 564, 203-219.

Bowers,M.B., Jr., Van,W.M. & Davis,L. (1971) Sexual behavior during L-dopa treatment for Parkinsonism. *Am.J.Psychiatry*, 127, 1691-1693.

Brackett,N.L. (1999) Semen retrieval by penile vibratory stimulation in men with spinal cord injury. *Hum.Reprod.Update.*, 5, 216-222.

Brailoiu,G.C., Dun,S.L., Brailoiu,E., Inan,S., Yang,J., Chang,J.K. & Dun,N.J. (2007) Nesfatin-1: distribution and interaction with a G protein-coupled receptor in the rat brain. *Endocrinology*, 148, 5088-5094.

Bressler,S.C. & Baum,M.J. (1996) Sex comparison of neuronal fos immunoreactivity in the rat vomeronasal projection circuit after chemosensory stimulation. *Neuroscience*, 71, 1063-1072.

Bridges,T.E., Hillhouse,E.W. & Jones,M.T. (1976) The effect of dopamine on neurohypophysial hormone release in vivo and from the rat neural lobe and hypothalamus in vitro. *J.Physiol*, 260, 647-666.

Briganti,A., Salonia,A., Gallina,A., Suardi,N., Rigatti,P. & Montorsi,F. (2004) Emerging oral drugs for erectile dysfunction. *Expert.Opin.Emerg.Drugs*, 9, 179-189.

Brioni,J.D., Moreland,R.B., Cowart,M., Hsieh,G.C., Stewart,A.O., Hedlund,P., Donnelly-Roberts,D.L., Nakane,M., Lynch,J.J., Kolasa,T., Polakowski,J.S., Osinski,M.A., Marsh,K., Andersson,K.E. & Sullivan,J.P. (2004) Activation of dopamine D-4 receptors by ABT-724 induces penile erection in rats. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 6758-6763.

Brock,J.W., Farooqui,S., Ross,K. & Prasad,C. (1992) Localization of dopamine D2 receptor protein in rat brain using polyclonal antibody. *Brain Res.*, 578, 244-250.

Brunton,P.J., Bales,J. & Russell,J.A. (2006) Neuroendocrine stress but not feeding responses to centrally administered neuropeptide Y are suppressed in pregnant rats. *Endocrinology*, 147, 3737-3745.

Buijs,R.M., Geffard,M., Pool,C.W. & Hoorneman,E.M. (1984) The dopaminergic innervation of the supraoptic and paraventricular nucleus. A light and electron microscopical study. *Brain Res.*, 323, 65-72.

Caldwell,J.D., Jirikowski,G.F., Greer,E.R. & Pedersen,C.A. (1989) Medial Preoptic Area Oxytocin and Female Sexual Receptivity. *Behavioral Neuroscience*, 103, 655-&.

Caldwell,J.D., Johns,J.M., Faggin,B.M., Senger,M.A. & Pedersen,C.A. (1994) Infusion of An Oxytocin Antagonist Into the Medial Preoptic Area Prior to Progesterone Inhibits Sexual Receptivity and Increases Rejection in Female Rats. *Hormones and Behavior*, 28, 288-302.

Cameron,J.L., Pomerantz,S.M., Layden,L.M. & Amico,J.A. (1992) Dopaminergic stimulation of oxytocin concentrations in the plasma of male and female monkeys by apomorphine and a D2 receptor agonist. *J.Clin.Endocrinol.Metab*, 75, 855-860.

Campbell,A. (2008) Attachment, aggression and affiliation: the role of oxytocin in female social behavior. *Biol Psychol.* 77(1):1-10.

Canteras,N.S., Simerly,R.B. & Swanson,L.W. (1995) Organization of Projections from the Medial Nucleus of the Amygdala - A Phal Study in the Rat. *Journal of Comparative Neurology*, 360, 213-245.

Caquineau,C., Leng,G., Guan,X.M.M., Jiang,M., Van der Ploeg,L. & Douglas,A.J. (2006) Effects of alpha-melanocyte-stimulating hormone on magnocellular oxytocin neurones and their activation at intromission in male rats. *Journal of Neuroendocrinology*, 18, 685-691.

Carmichael,M.S., Humbert,R., Dixen,J., Palmisano,G., Greenleaf,W. & Davidson,J.M. (1987) Plasma Oxytocin Increases in the Human Sexual-Response. *Journal of Clinical Endocrinology and Metabolism*, 64, 27-31.

Castelli,M.P., Piras,A.P., Melis,T., Succu,S., Sanna,F., Melis,M.R., Collu,S., Grazia,E.M., Diaz,G., Mackie,K. & Argiolas,A. (2007) Cannabinoid CB1 receptors in the paraventricular nucleus and central control of penile erection: immunocytochemistry, autoradiography and behavioral studies. *Neuroscience*, 147, 197-206.

Chapelle,P.A., Durand,J. & Lacert,P. (1980) Penile erection following complete spinal cord injury in man. *Br.J.Urol.*, 52, 216-219.

Charles,M.A. & McGinnis,M.Y. (1992) Effects of LY163502, a D2 dopaminergic agonist, on the sexual behavior of male rats. *Pharmacol.Biochem.Behav.*, 43, 1087-1092.

Chen,K., Swedlow,J.R., Grote,M., Sedat, J.W., Agard, D.A. (1995) The collection, processing and display of digital three-dimensional images of biological specimens. In *Handbook of Biological Confocal Microscopy*, 2nd edition, Pawley, JB., Ed.; Plenum Press, New York, 197-210.

Chen,K.K., Chan,J.Y., Chang,L.S., Chen,M.T. & Chan,S.H. (1992) Elicitation of penile erection following activation of the hippocampal formation in the rat. *Neurosci.Lett.*, 141, 218-222.

Chen,K.K., Chan,S.H.H., Chang,L.S. & Chan,J.Y.H. (1997) Participation of paraventricular nucleus of hypothalamus in central regulation of penile erection in the rat. *Journal of Urology*, 158, 238-244.

Chen,K.K., Chan,J.Y.H. & Chang,L.S. (1999) Dopaminergic neurotransmission at the paraventricular nucleus of hypothalamus in central regulation of penile erection in the rat. *Journal of Urology*, 162, 237-242.

Cho,M.M., DeVries,A.C.,Williams,J.R. & Carter,C.S. (1999) The effects of oxytocin and vasopressin on partner preferences in male and female prairie voles (Microtus ochrogaster). *Behav Neurosci.* 113(5):1071-9.

Clancy,A.N., Zumpe,D. & Michael,R.P. (2000) Estrogen in the medial preoptic area of male rats facilitates copulatory behavior. *Horm Behav.* 38(2):86-93.

Clement,P., Bernabe,J., Denys,P., Alexandre,L. & Giuliano,F. (2007) Ejaculation induced by i.c.v. injection of the preferential dopamine D(3) receptor agonist 7-hydroxy-2-(di-N-propylamino)tetralin in anesthetized rats. *Neuroscience*, 145, 605-610.

Collins,G.T., Witkin,J.M., Newman,A.H., Svensson,K.A., Grundt,P., Cao,J. & Woods,J.H. (2005) Dopamine agonist-induced yawning in rats: a dopamine D3 receptor-mediated behavior. *J.Pharmacol.Exp.Ther.*, 314, 310-319.

Condes-Lara,M., Martinez-Lorenzana,G., Rojas-Piloni,G. & Rodriguez-Jimenez,J. (2007) Branched oxytocinergic innervations from the paraventricular hypothalamic nuclei to superficial layers in the spinal cord. *Brain Res.*, 1160, 20-29.

Conrad,L.C.A. & Pfaff,D.W. (1976) Efferents from Medial Basal Forebrain and Hypothalamus in Rat: Autoradiographic Study of Medial Preoptic Area. *Journal of Comparative Neurology*, 169, 185-219.

Contreras,J.L. & Agmo,A. (1993) Sensory control of the male rat's copulatory thrusting patterns. *Behav.Neural Biol.*, 60, 234-240.

Coolen,L.M., Peters,H.J.P.W. & Veening,J.G. (1996) Fos immunoreactivity in the rat brain following consummatory elements of sexual behavior: A sex comparison. *Brain Research*, 738, 67-82.

Coolen,L.M., Allard,J., Truitt,W.A. & McKenna,K.E. (2004) Central regulation of ejaculation. *Physiol Behav.*, 83, 203-215.

Coons, A.H. & Kaplan,M.H. (1949) Localisation of antigen in tissue cells. *J. Exp. Medicine.*, 91, 1-13.

Corbin,J.D., Francis,S.H. & Webb,D.J. (2002) Phosphodiesterase type 5 as a pharmacologic target in erectile dysfunction. *Urology*, 60, 4-11.

Croci,T., Cecchi,R., Marini,P., Rouget,C., Viviani,N., Germain,G., Guagnini,F., Fradin,Y., Descamps,L., Pascal,M., Advenier,C., Breuiller-Fouche,M., Leroy,M.J. & Bardou,M. (2007) In vitro and in vivo pharmacological characterization of ethyl-4-[trans-4-[((2S)-2-hydroxy-3-[4-hydroxy-3[(methylsulfonyl)amino]-phenoxy]p ropyl) amino]cyclohexyl]benzoate hydrochloride (SAR150640), a new potent and selective human beta3-adrenoceptor agonist for the treatment of preterm labor. *J.Pharmacol.Exp.Ther.*, 321, 1118-1126.

Croze,E., Usacheva,A., Asarnow,D., Minshall,R.D., Perez,H.D. & Colamonici,O. (2000) Receptor for activated C-kinase (RACK-1), a WD motif-containing protein, specifically associates with the human type I IFN receptor. *J.Immunol.*, 165, 5127-5132.

Curran,E.J. & Watson,S.J., Jr. (1995) Dopamine receptor mRNA expression patterns by opioid peptide cells in the nucleus accumbens of the rat: a double in situ hybridization study. *J.Comp Neurol.*, 361, 57-76.

Curran,T. & Morgan,J.I. (1995) Fos: an immediate-early transcription factor in neurons. *J.Neurobiol.*, 26, 403-412.

Cushing,B.S. & Carter,C.S. (2000) Peripheral pulses of oxytocin increase partner preferences in female, but not male, prairie voles. *Horm Behav.* 37(1):49-56.

Czyrak,A., Chocyk,A., Mackowiak,M., Fijal,K. & Wedzony,K. (2000) Distribution of dopamine D1 receptors in the nucleus paraventricularis of the hypothalamus in rats: an immunohistochemical study. *Brain Res.Mol.Brain Res.*, 85, 209-217.

D'Aquila,P.S., Panin,F., Cossu,M., Peana,A.T. & Serra,G. (2003) Dopamine D1 receptor agonists induce penile erections in rats. *Eur.J.Pharmacol.*, 460, 71-74.

Damsma,G., Wenkstern,D., Pfaus,J.G., Phillips,A.G. & Fibiger,H.C. (1992) Sexual-Behavior Increases Dopamine Transmission in the Nucleus-Accumbens and Striatum of Male-Rats - Comparison with Novelty and Locomotion. *Behavioral Neuroscience*, 106, 181-191.

de Jong,T.R., Veening,J.G., Olivier,B. & Waldinger,M.D. (2007) Oxytocin involvement in SSRI-induced delayed ejaculation: A review of animal studies. *Journal of Sexual Medicine*, 4, 14-28.

de Kock,C.P., Wierda,K.D., Bosman,L.W., Min,R., Koksma,J.J., Mansvelder,H.D., Verhage,M. & Brussaard,A.B. (2003) Somatodendritic secretion in oxytocin neurons is upregulated during the female reproductive cycle. *J.Neurosci.*, 23, 2726-2734.

Dean,B., Pavey,G., Scarr,E., Goeringer,K. & Copolov,D.L. (2004) Measurement of dopamine D2-like receptors in postmortem CNS and pituitary: differential regional changes in schizophrenia. *Life Sci.*, 74, 3115-3131.

Dean,B. & Scarr,E. (2004) Antipsychotic drugs: evolving mechanisms of action with improved therapeutic benefits. *Curr.Drug Targets CNS.Neurol.Disord.*, 3, 217-225.

Decavel,C., Geffard,M. & Calas,A. (1987) Comparative study of dopamine- and noradrenaline-immunoreactive terminals in the paraventricular and supraoptic nuclei of the rat. *Neurosci.Lett.*, 77, 149-154.

Defagot,M.C., Malchiodi,E.L., Villar,M.J. & Antonelli,M.C. (1997) Distribution of D4 dopamine receptor in rat brain with sequence-specific antibodies. *Brain Res.Mol.Brain Res.*, 45, 1-12.

den Hertog,C.E., de Groot,A.N. & van Dongen,P.W. (2001) History and use of oxytocics. *Eur.J.Obstet.Gynecol.Reprod.Biol.*, 94, 8-12.

Dennison,E., Bain,P.A., Bartke,A. & Meliska,C.J. (1996) Systemically administered gonadotrophin-releasing hormone enhances copulatory behaviour in castrated, testosterone-treated hyperprolactinaemic male rats. *Int.J.Androl*, 19, 253-259.

Diaz,J., Levesque,D., Lammers,C.H., Griffon,N., Martres,M.P., Schwartz,J.C. & Sokoloff,P. (1995) Phenotypical characterization of neurons expressing the dopamine D3 receptor in the rat brain. *Neuroscience*, 65, 731-745.

Dinsmore,W. (2004) Treatment of erectile dysfunction. *Int.J.STD AIDS*, 15, 215-221.

Doherty,P.C. & Wisler,P.A. (1994) Stimulatory effects of quinelorane on yawning and penile erection in the rat. *Life Sci.*, 54, 507-514.

Dominguez,J., Riolo,J.V., Xu,Z. & Hull,E.M. (2001) Regulation by the medial amygdala of copulation and medial preoptic dopamine release. *J.Neurosci.*, 21, 349-355.

Dominguez,J.M. & Hull,E.M. (2005) Dopamine, the medial preoptic area, and male sexual behavior. *Physiology & Behavior*, 86, 356-368.

Donatucci,C.F. (2006) Etiology of ejaculation and pathophysiology of premature ejaculation. *J.Sex Med.*, 3 Suppl 4, 303-308.

Dong,H.W. & Swanson,L.W. (2006) Projections from bed nuclei of the stria terminalis, magnocellular nucleus: Implications for cerebral hemisphere regulation of micturition, defecation, and penile erection. *Journal of Comparative Neurology*, 494, 108-141.

Eaton,R.C., Markowski,V.P., Lumley,L.A., Thompson,J.T., Moses,J. & Hull,E.M. (1991) D2 receptors in the paraventricular nucleus regulate genital responses and copulation in male rats. *Pharmacol.Biochem.Behav.*, 39, 177-181.

Eaton,R.C., Markowski,V.P., Lumley,L.A., Thompson,J.T., Moses,J. & Hull,E.M. (1991) D2 Receptors in the Paraventricular Nucleus Regulate Genital Responses and Copulation in Male-Rats. *Pharmacology Biochemistry and Behavior*, 39, 177-181.

Ebner,K., Wotjak,C.T., Landgraf,R. & Engelmann,M. (2000) A single social defeat experience selectively stimulates the release of oxytocin, but not vasopressin, within the septal brain area of male rats. *Brain Research*, 872, 87-92.

Edwards,D.A. & Isaacs,S. (1991) Zona incerta lesions: effects on copulation, partner-preference and other socio-sexual behaviors. *Behav.Brain Res.*, 44, 145-150.

Eliasson,M. & Meyerson,B.J. (1976) Comparison of the action of lysergic acid diethylamide and apomorphine on the copulatory response in the female rat. *Psychopharmacology (Berl)*. 49(3):301-6.

El-Din,M.M., Senbel,A.M., Daabees,T.T. & Sharabi,F.M. (2007) Peripheral modulation of dopaminergic receptors affects erectile responses in rats. *Basic & Clinical Pharmacology & Toxicology*, 100, 225-232.

Engelmann M, Ebner K, Landgraf R, Holsboer F, Wotjak1 CT. (1999) Emotional stress triggers intrahypothalamic but not peripheral release of oxytocin in male rats. *J Neuroendocrinol.* 11(11):867-72.

Etgen AM, Karkanias GB. (1994) Estrogen regulation of noradrenergic signaling in the hypothalamus. *Psychoneuroendocrinology*. 19(5-7):603-10.

Everitt,B.J., Fuxe,K., Hökfelt,F.T. & Jonsson,G. (1975) Role of monoamines in the control by hormones of sexual receptivity in the female rat. *J Comp Physiol Psychol.* 89(6):556-72.

Everitt,B.J. (1990) Sexual motivation: a neural and behavioural analysis of the mechanisms underlying appetitive and copulatory responses of male rats. *Neurosci.Biobehav.Rev.*, 14, 217-232.

Ferguson,J.N., Young,L.J., Hearn,E.F., Matzuk,M.M., Insel,T.R. & Winslow,J.T. (2000) Social amnesia in mice lacking the oxytocin gene. *Nat Genet.* 25(3):284-8.

Ferguson,J.N., Aldag,J.M., Insel,T.R. & Young,L.J. (2001) Oxytocin in the medial amygdala is essential for social recognition in the mouse. *J.Neurosci.*, 21, 8278-8285.

Ferguson,J.N., Young,L.J. & Insel,T.R. (2002) The neuroendocrine basis of social recognition. *Front Neuroendocrinol.*, 23, 200-224.

Ferrari,F. & Claudi,F. (1991) Behavioural evidence for central D-2 dopamine receptor agonistic effect by some 2-(fluorohydroxyphenyl)ethylamines. *Pharmacol.Biochem.Behav.*, 38, 131-134.

Ferrini,M., Wang,C., Swerdloff,R.S., Sinha Hikim,A.P., Rajfer,J. & Gonzalez-Cadavid,N.F. (2001) Aging-related increased expression of inducible nitric oxide synthase and cytotoxicity markers in rat hypothalamic regions associated with male reproductive function. *Neuroendocrinology*, 74, 1-11.

Ferrini,M.G., Magee,T.R., Vernet,D., Rajfer,J. & Gonzalez-Cadavid,N.F. (2003) Penile neuronal nitric oxide synthase and its regulatory proteins are present in hypothalamic and spinal cord regions involved in the control of penile erection. *J.Comp Neurol.*, 458, 46-61.

Foreman, M.M. & Moss,R.L. (1979) Role of hypothalamic dopaminergic receptors in the control of lordosis behaviour in the female rat. *Physiol Behav.* 22(2):283-9.

Foreman,M.M. & Hall,J.L. (1987) Effects of D2-dopaminergic receptor stimulation on male rat sexual behavior. *J.Neural Transm.*, 68, 153-170.

Foresta,C., Caretta,N., Rossato,M., Garolla,A. & Ferlin,A. (2004) Role of androgens in erectile function. *J.Urol.*, 171, 2358-62.

Freund-Mercier,M.J., Stoeckel,M.E., Palacios,J.M., Pazos,A., Reichhart,J.M., Porte,A. & Richard,P. (1987) Pharmacological characteristics and anatomical distribution of [3H]oxytocin-binding sites in the Wistar rat brain studied by autoradiography. *Neuroscience*, 20, 599-614.

Freund-Mercier,M.J., Stoeckel,M.E. & Klein,M.J. (1994) Oxytocin receptors on oxytocin neurones: histoautoradiographic detection in the lactating rat. *J Physiol.* 480 (Pt 1):155-61.

Fuchs,A.R., Fields,M.J., Freidman,S., Shemesh,M. & Ivell,R. (1995) Oxytocin and the timing of parturition. Influence of oxytocin receptor gene expression, oxytocin secretion, and oxytocin-induced prostaglandin F2 alpha and E2 release. *Adv Exp Med Biol.* 395:405-20.

Gao,X., Chen,H., Schwarzschild,M.A., Glasser,D.B., Logroscino,G., Rimm,E.B. & Ascherio,A. (2007) Erectile function and risk of Parkinson's disease. *American Journal of Epidemiology*, 166, 1446-1450.

Gazi,L., Schoeffter,P., Nunn,C., Croskery,K., Hoyer,D. & Feuerbach,D. (2000) Cloning, expression, functional coupling and pharmacological characterization of the rat dopamine D4 receptor. *Naunyn Schmiedebergs Arch.Pharmacol.*, 361, 555-564.

Gerstenberg,T.C., Levin,R.J. & Wagner,G. (1990) Erection and ejaculation in man. Assessment of the electromyographic activity of the bulbocavernosus and ischiocavernosus muscles 19. *Br.J.Urol.*, 65, 395-402.

Gies,U. & Theodosis,D.T. (1994) Synaptic plasticity in the rat supraoptic nucleus during lactation involves GABA innervation and oxytocin neurons: a quantitative immunocytochemical analysis. *J.Neurosci.*, 14, 2861-2869.

Gimpl,G. & Fahrenholz,F. (2001) The oxytocin receptor system: structure, function, and regulation. *Physiol Rev.* 81(2):629-83.

Giraldi,A., Marson,L., Nappi,R., Pfaus,J., Traish,A.M., Vardi,Y. & Goldstein,I. (2004) Physiology of female sexual function: animal models. *J Sex Med.* 1(3):237-53.

Giuliano,F., Bernabe,J., Jardin,A. & Rousseau,J.P. (1993) Antierectile role of the sympathetic nervous system in rats. *J.Urol.*, 150, 519-524.

Giuliano,F., Rampin,O., Brown,K., Courtois,F., Benoit,G. & Jardin,A. (1996) Stimulation of the medial preoptic area of the hypothalamus in the rat elicits increases in intracavernous pressure. *Neuroscience Letters*, 209, 1-4.

Giuliano,F., Bernabe,J., Brown,K., Droupy,S., Benoit,G. & Rampin,O. (1997) Erectile response to hypothalamic stimulation in rats: role of peripheral nerves. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, 42, R1990-R1997.

Giuliano,F., Rampin,O. & Jardin,A. (1997) [Physiology of erection]. *Rev.Med.Interne*, 18 Suppl 1, 3s-9s.

Giuliano,F. & Rampin,O. (2000) Central neural regulation of penile erection. *Neuroscience and Biobehavioral Reviews*, 24, 517-533.

Giuliano,F. & Allard,J. (2001) Dopamine and male sexual function. *Eur.Urol.*, 40, 601-608.

Giuliano,F., Bernabe,J., McKenna,K., Longueville,F. & Rampin,O. (2001) Spinal proerectile effect of oxytocin in anesthetized rats. *Am.J.Physiol Regul.Integr.Comp Physiol*, 280, R1870-R1877.

Giuliano,F., Allard,J., Rampin,O., Droupy,S., Benoit,G., Alexandre,L. & Bernabe,J. (2001) Spinal proerectile effect of apomorphine in the anesthetized rat. *Int.J.Impot.Res.*, 13, 110-115.

Giuliano,F., Allard,J., Rampin,O., Droupy,S., Benoit,G., Alexandre,L. & Bernabe,J. (2002) Pro-erectile effect of systemic apomorphine: Existence of a spinal site of action. *Journal of Urology*, 167, 402-406.

Giuliano,F. & Rampin,O. (2004) Neural control of erection. *Physiol Behav.*, 83, 189-201.

Giuliano,F. (2004) Control of penile erection by the melanocortinergic system: experimental evidences and therapeutic perspectives. *J.Androl*, 25, 683-691.

Glander,H.J., Haidl,G., Kohn,F.M., Ochsendorf,F., Paasch,U. & Schuppe,H.C. (2007) Andrology. *J.Dtsch.Dermatol.Ges.*, 5, 924-933.

Gobrogge,K.L., Liu,Y., Jia,X. & Wang,Z. (2007) Anterior hypothalamic neural activation and neurochemical associations with aggression in pair-bonded male prairie voles. *J Comp Neurol.* 502(6):1109-22

Gonzalez-Cadavid,N.F., Burnett,A.L., Magee,T.R., Zeller,C.B., Vernet,D., Smith,N., Gitter,J. & Rajfer,J. (2000) Expression of penile neuronal nitric oxide synthase variants in the rat and mouse penile nerves. *Biol.Reprod.*, 63, 704-714.

Grace,A.A. (1993) Cortical regulation of subcortical dopamine systems and its possible relevance to schizophrenia. *J.Neural Transm.Gen.Sect.*, 91, 111-134.

Granneman,J.G., Moore,H.P., Granneman,R.L., Greenberg,A.S., Obin,M.S. & Zhu,Z. (2007) Analysis of lipolytic protein trafficking and interactions in adipocytes. *J.Biol.Chem.*, 282, 5726-5735.

Gray,T.S., Carney,M.E. & Magnuson,D.J. (1989) Direct Projections from the Central Amygdaloid Nucleus to the Hypothalamic Paraventricular Nucleus - Possible Role in Stress-Induced Adrenocorticotropin Release. *Neuroendocrinology*, 50, 433-446.

Greco,B., Edwards,D.A., Michael,R.P. & Clancy,A.N. (1998) Androgen receptors and estrogen receptors are colocalized in male rat hypothalamic and limbic neurons that express Fos immunoreactivity induced by mating. *Neuroendocrinology*, 67, 18-28.

Greiner,K.A. & Weigel,J.W. (1996) Erectile dysfunction. *Am.Fam.Physician*, 54, 1675-1682.

Griffith,E.R., Tomko,M.A. & Timms,R.J. (1973) Sexual function in spinal cord-injured patients: a review. *Arch.Phys.Med.Rehabil.*, 54, 539-543.

Gurevich,E.V. & Joyce,J.N. (1999) Distribution of dopamine D3 receptor expressing neurons in the human forebrain: comparison with D2 receptor expressing neurons. *Neuropsychopharmacology*, 20, 60-80.

Hallbeck,M., Larhammar,D. & Blomqvist,A. (2001) Neuropeptide expression in rat paraventricular hypothalamic neurons that project to the spinal cord. *J.Comp Neurol.*, 433, 222-238.

Hamburger-Bar,R. & Rigter,H. (1975) Apomorphine: facilitation of sexual behaviour in female rats. *Eur J Pharmacol.* 32(02):357-60.

Hancock,M.B. & Peveto,C.A. (1979) A preganglionic autonomic nucleus in the dorsal gray commissure of the lumbar spinal cord of the rat. *J.Comp Neurol.*, 183 , 65-72.

Hancock,M.B. & Peveto,C.A. (1979) Preganglionic neurons in the sacral spinal cord of the rat: an HRP study. *Neurosci.Lett.*, 11, 1-5.

Hansen,S., Kohler,C., Goldstein,M. & Steinbusch,H.V. (1982) Effects of ibotenic acid-induced neuronal degeneration in the medial preoptic area and the lateral hypothalamic area on sexual behavior in the male rat. *Brain Res.*, 239, 213-232.

Hara,K. & Harris,R.A. (2002) The anesthetic mechanism of urethane: the effects on neurotransmitter-gated ion channels. *Anesth.Analg.*, 94, 313-8, table.

Harris,V.S. & Sachs,B.D. (1975) Copulatory-Behavior in Male Rats Following Amygdaloid Lesions. *Brain Research* , 86, 514-518.

Harsing,L.G., Jr. & Zigmond,M.J. (1997) Influence of dopamine on GABA release in striatum: evidence for D1-D2 interactions and non-synaptic influences. *Neuroscience*, 77, 419-429.

Hart,B.L., & Haugen,C.M. (1968) Activation of sexual reflexes in male rats by spinal implementation of testosterone. *Physiol Behav*, 3, 735-738.

Hawthorn,J., Ang,V.T.Y. & Jenkins,J.S. (1985) Effects of Lesions in the Hypothalamic Paraventricular, Supraoptic and Suprachiasmatic Nuclei on Vasopressin and Oxytocin in Rat-Brain and Spinal-Cord. *Brain Research*, 346, 51-57.

Hayes,E.S. & Adaikan,P.G. (2002) The effects of 5HT(1) agonists on erection in rats in vivo and rabbit corpus cavernosum in vitro. *Int.J.Impot.Res.*, 14, 205-212.

Heaton,J.P. & Varrin,S.J. (1994) Effects of castration and exogenous testosterone supplementation in an animal model of penile erection. *J.Urol.*, 151, 797-800.

Heaton,J.P.W. (2000) Central neuropharmacological agents and mechanisms in erectile dysfunction: the role of dopamine. *Neuroscience and Biobehavioral Reviews*, 24, 561-569.

Herman,J.P., Ostrander,M.M., Mueller,N.K. & Figueiredo,H. (2005) Limbic system mechanisms of stress regulation: Hypothalamo-pituitary-adrenocortical axis. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, 29, 1201-1213.

Hirota,K., Roth,S.H., Fujimura,J., Masuda,A. & Ito,Y. (1998) GABAergic mechanisms in the action of general anesthetics. *Toxicol.Lett.*, 100-101, 203-207.

Holmes,G.M., Chapple,W.D., Leipheimer,R.E. & Sachs,B.D. (1991) Electromyographic analysis of male rat perineal muscles during copulation and reflexive erections. *Physiol Behav.*, 49, 1235-1246.

Holmes,G.M. & Sachs,B.D. (1991) The ejaculatory reflex in copulating rats: normal bulbospongiosus activity without apparent urethral stimulation. *Neurosci.Lett.*, 125, 195-197.

Holstege,J.C., VanDijken,H., Buijs,R.M., Goedknegt,H., Gosens,T. & Bongers,C.M.H. (1996) Distribution of dopamine immunoreactivity in the rat, cat, and monkey spinal cord. *Journal of Comparative Neurology*, 376, 631-652.

Honda,K., Yanagimoto,M., Negoro,H., Narita,K., Murata,T. & Higuchi,T. (1999) Excitation of oxytocin cells in the hypothalamic supraoptic nucleus by electrical stimulation of the dorsal penile nerve and tactile stimulation of the penis in the rat. *Brain Res.Bull.*, 48, 309-313.

Hsieh,G.C., Hollingsworth,P.R., Martino,B., Chang,R.J., Terranova,M.A., O'Neill,A.B., Lynch,J.J., Moreland,R.B., Donnelly-Roberts,D.L., Kolasa,T., Mikusa,J.P., McVey,J.M., Marsh,K.C., Sullivan,J.P. & Brioni,J.D. (2004) Central mechanisms regulating penile erection in conscious rats: The dopaminergic systems related to the proerectile effect of apomorphine. *Journal of Pharmacology and Experimental Therapeutics*, 308, 330-338.

Huber,D., Veinante,P. & Stoop,R. (2005) Vasopressin and oxytocin excite distinct neuronal populations in the central amygdala. *Science*. 308(5719):245-8.

Hughes,A.M., Everitt,B.J., Lightman,S.L. & Todd,K. (1987) Oxytocin in the Central-Nervous-System and Sexual-Behavior in Male-Rats
21. *Brain Research*, 414 , 133-137.

Hull,E.M., Bitran,D., Pehek,E.A., Warner,R.K., Band,L.C. & Holmes,G.M. (1986) Dopaminergic control of male sex behavior in rats: effects of an intracerebrally-infused agonist. *Brain Res.*, 370, 73-81.

Hull,E.M., Eaton,R.C., Markowski,V.P., Moses,J., Lumley,L.A. & Loucks,J.A. (1992) Opposite Influence of Medial Preoptic D1 and D2 Receptors on Genital Reflexes - Implications for Copulation. *Life Sciences*, 51, 1705-1713.

Hull,E.M., Eaton,R.C., Moses,J. & Lorrain,D. (1993) Copulation increases dopamine activity in the medial preoptic area of male rats. *Life Sci.*, 52, 935-940.

Hull,E.M., Du,J., Lorrain,D.S. & Matuszewich,L. (1995) Extracellular dopamine in the medial preoptic area: implications for sexual motivation and hormonal control of copulation. *J.Neurosci.* , 15, 7465-7471.

Hull,E.M., Du,J., Lorrain,D.S. & Matuszewich,L. (1997) Testosterone, preoptic dopamine, and copulation in male rats. *Brain Res.Bull.*, 44, 327-333.

Hull,E.M., Lorrain,D.S., Du,J.F., Matuszewich,L., Lumley,L.A., Putnam,S.K. & Moses,J. (1999) Hormone-neurotransmitter interactions in the control of sexual behavior. *Behavioural Brain Research*, 105, 105-116.

Hull,E.M., Muschamp,J.W. & Sato,S. (2004) Dopamine and serotonin: influences on male sexual behavior. *Physiol Behav.*, 83, 291-307.

Hull,E.M. & Dominguez,J.M. (2006) Getting his act together: Roles of glutamate, nitric oxide, and dopamine in the medial preoptic area. *Brain Research*, 1126, 66-75.

Hull,E.M. & Dominguez,J.M. (2007) Sexual behavior in male rodents. *Horm.Behav.*, 52, 45-55.

Insel,T.R. (1997a) A neurobiological basis of social attachment. *American Journal of Psychiatry*, 154, 726-735.

Insel,T.R., Young,L. & Wang,Z. (1997b) Central oxytocin and reproductive behaviours. *Rev.Reprod.*, 2, 28-37.

Ishizuka,O., Gu,B.J., Nishizawa,O., Mizusawa,H. & Andersson,K.E. (2002) Effect of apomorphine on intracavernous pressure and blood pressure in conscious, spinalized rats. *Int.J.Impot.Res.*, 14, 128-132.

Jenner,P. (2003) Dopamine agonists, receptor selectivity and dyskinesia induction in Parkinson's disease. *Curr.Opin.Neurol.*, 16 Suppl 1, S3-S7.

Jones,G.H. & Robbins,T.W. (1992) Differential-Effects of Mesocortical, Mesolimbic, and Mesostriatal Dopamine Depletion on Spontaneous, Conditioned, and Drug-Induced Locomotor-Activity. *Pharmacology Biochemistry and Behavior*, 43, 887-895.

Jordan,C. (1997) Androgen receptor (AR) immunoreactivity in rat pudendal motoneurons: implications for accessory proteins. *Horm.Behav.*, 32, 1-10.

Jourdain,P., Dupouy,B., Bonhomme,R., Poulain,D.A., Israel,J.M. & Theodosis,D.T. (1999) Visualization of local afferent inputs to magnocellular oxytocin neurons in vitro. *Eur.J.Neurosci.*, 11, 1960-1972.

Kaminetsky J. (2008) Epidemiology and pathophysiology of male sexual dysfunction. *Int J Impot Res*. 20 Suppl 1:S3-10.

Kendrick,K.M. (2000) Oxytocin, motherhood and bonding. *Experimental Physiology*, 85, 111S-124S.

Kendrick,K.M. (2004) The neurobiology of social bonds. *Journal of Neuroendocrinology*, 16, 1007-1008.

Khan,Z.U., Gutierrez,A., Martin,R., Penafiel,A., Rivera,A. & De La,C.A. (1998) Differential regional and cellular distribution of dopamine D2-like receptors: an immunocytochemical study of subtype-specific antibodies in rat and human brain. *J.Comp Neurol.*, 402, 353-371.

Kimura,T., Azuma,C., Saji,F., Takemura,M., Tokugawa,Y., Miki,M., Ono,M., Mori,K &,Tanizawa,O. (1992) Estimation by an electrophysiological method of the expression of oxytocin receptor mRNA in human myometrium during pregnancy. *J Steroid Biochem Mol Biol*. 42(3-4):253-8.

Kimura,Y., Naitou,Y., Wanibuchi,F. & Yamaguchi,T. (2006) Characterization of intracavernous pressure increase induced by Ym348, a novel 5-HT2C receptor agonist, in anesthetized rats. *J.Urol.*, 175, 1953-1957.

Kippin,TE., Cain,SW. & Pfaus, JG. (2003) Estrous odors and sexually conditioned neutral odors activate separate neural pathways in the male rat. *Neuroscience*, 117(4):971-9.

Kippin,T.E., Sotiropoulos,V., Badih,J. & Pfaus,J.G. (2004) Opposing roles of the nucleus accumbens and anterior lateral hypothalamic area in the control of sexual behaviour in the male rat. *European Journal of Neuroscience*, 19, 698-704.

Kirchgessner,A.L., Sclafani,A. & Nilaver,G. (1988) Histochemical identification of a PVN-hindbrain feeding pathway. *Physiol Behav.*, 42, 529-543.

Kiss,A. & Mikkelsen,J.D. (2005) Oxytocin--anatomy and functional assignments: a minireview. *Endocr.Regul.*, 39, 97-105.

Kiss,A., Soderman,A., Bundzikova,J., Pirnik,Z. & Mikkelsen,J.D. (2006) Zolpidem, a selective GABA(A) receptor alpha1 subunit agonist, induces comparable Fos expression in oxytocinergic neurons of the hypothalamic paraventricular and accessory but not supraoptic nuclei in the rat. *Brain Res.Bull.*, 71, 200-207.

Kita,I., Yoshida,Y. & Nishino,S. (2006) An activation of parvocellular oxytocinergic neurons in the paraventricular nucleus in oxytocin-induced yawning and penile erection. *Neuroscience Research*, 54, 269-275.

Kombian,S.B., Zidichouski,J.A. & Pittman,Q.J. (1996) GABAB receptors presynaptically modulate excitatory synaptic transmission in the rat supraoptic nucleus in vitro. *J.Neurophysiol.*, 76, 1166-1179.

Kondo,Y. (1992) Lesions of the Medial Amygdala Produce Severe Impairment of Copulatory-Behavior in Sexually Inexperienced Male-Rats. *Physiology & Behavior*, 51, 939-943.

Kondo,Y., Sachs,B.D. & Sakuma,Y. (1998) Importance of the medial amygdala in rat penile erection evoked by remote stimuli from estrous females (vol 88, pg 153, 1997). *Behavioural Brain Research*, 91, 213.

Kondo,Y. & Sachs,B.D. (2002) Disparate effects of small medial amygdala lesions on noncontact erection, copulation, and partner preference. *Physiology & Behavior*, 76, 443-447.

Koyama,Y., Satou,M., Oka,Y. & Ueda,K. (1984) Involvement of the Telencephalic Hemispheres and the Preoptic Area in Sexual-Behavior of the Male Goldfish, Carassius-Auratus - A Brain-Lesion Study. *Behavioral and Neural Biology*, 40, 70-86.

Kruger,T.H.C., Hartmann,U. & Schedlowski,M. (2005) Prolactinergic and dopaminergic mechanisms underlying sexual arousal and orgasm in humans. *World Journal of Urology*, 23, 130-138.

Kruger,T.H.C., Schiffer,B., Eikermann,M., Haake,P., Gizewski,E. & Schedlowski,M. (2006) Serial neurochemical measurement of cerebrospinal fluid during the human sexual response cycle. *European Journal of Neuroscience*, 24, 3445-3452.

Kudwa,A.E., Dominguez-Salazar,E., Cabrera,D.M., Sibley,D.R. & Rissman,E.F. (2005) Dopamine D5 receptor modulates male and female sexual behavior in mice. *Psychopharmacology (Berl)*, 180, 206-214.

Kupelian,V., Shabsigh,R., Travison,T.G., Page,S.T., Araujo,A.B. & McKinlay,J.B. (2006) Is there a relationship between sex hormones and erectile dysfunction? Results from the Massachusetts Male Aging Study. *J.Urol.*, 176, 2584-2588.

Lambert,R.C., Dayanithi,G., Moos,F.C. & Richard,P. (1994) A rise in the intracellular Ca2+ concentration of isolated rat supraoptic cells in response to oxytocin. *J.Physiol*, 478 (Pt 2), 275-287.

Landgraf,R. (1981) Simultaneous measurement of arginine vasopressin and oxytocin in plasma andneurohypophyses by radioimmunoassay. *Endokrinologie*. 78(2-3):191-204.

Landgraf,R., Malkinson,T., Horn,T., Veale,W.L., Lederis,K. & Pittman,Q.J. (1990) Release of vasopressin and oxytocin by paraventricular stimulation in rats. *Am.J.Physiol*, 258, R155-R159.

Landtblom,A.M. (2006) Treatment of erectile dysfunction in multiple sclerosis. *Expert.Rev.Neurother.*, 6, 931-935.

Larsen,P.J., Hayschmidt,A. & Mikkelsen,J.D. (1994) Efferent Connections from the Lateral Hypothalamic Region and the Lateral Preoptic Area to the Hypothalamic Paraventricular Nucleus of the Rat. *Journal of Comparative Neurology*, 342, 299-319.

Lavoisier,P., Courtois,F., Proulx,J., Durand,L.G. & de,C.F. (1990) Validation of a non-invasive device to measure intracavernous pressure as an index of penile rigidity. *Br.J.Urol.*, 65, 624-628.

Le,M.C. & Bloch,B. (1996) Expression of the D3 dopamine receptor in peptidergic neurons of the nucleus accumbens: comparison with the D1 and D2 dopamine receptors. *Neuroscience*, 73, 131-143.

Le,M.C. & Gaspar,P. (1998) Subpopulations of cortical GABAergic interneurons differ by their expression of D1 and D2 dopamine receptor subtypes. *Brain Res.Mol.Brain Res.*, 58, 231-236.

Lehman, MN. & Winans, SS. (1982) Vomeronasal and olfactory pathways to the amygdala controlling male hamster sexual behavior: autoradiographic and behavioral analyses. *Brain Res.*, 240(1):27-41

Leng,G., Brown,C.H. & Russell,J.A. (1999) Physiological pathways regulating the activity of magnocellular neurosecretory cells. *Prog.Neurobiol.*, 57, 625-655.

Levant,B. (1997) The D3 dopamine receptor: neurobiology and potential clinical relevance. *Pharmacol.Rev.*, 49, 231-252.

Levey,A.I., Hersch,S.M., Rye,D.B., Sunahara,R.K., Niznik,H.B., Kitt,C.A., Price,D.L., Maggio,R., Brann,M.R., Ciliax,B.J. & . (1993) Localization of D1 and D2 dopamine receptors in brain with subtype-specific antibodies. *Proc.Natl.Acad.Sci.U.S.A*, 90, 8861-8865.

Lim,M.M., Wang,Z., Olazábal,D.E., Ren,X., Terwilliger,E.F. & Young,L.J. (2004) Enhanced partner preference in a promiscuous species by manipulating the expression of a single gene. *Nature*. 429(6993):754-7.

Lin,S.H., Kiyohara,T. & Sun,B. (2003) Maternal behavior: activation of the central oxytocin receptor system in parturient rats? *Neuroreport*, 14, 1439-1444.

Lin,Y.M. & Lin,J.S. (1996) The rabbit as an intracavernous injection study model. *Urol.Res.*, 24, 27-32.

Liposits,Z. (1993) Ultrastructure of Hypothalamic Paraventricular Neurons. *Critical Reviews in Neurobiology*, 7, 89-162.

Liu,Y.C., Salamone,J.D. & Sachs,B.D. (1997) Impaired sexual response after lesions of the paraventricular nucleus of the hypothalamus in male rats. *Behavioral Neuroscience*, 111, 1361-1367.

Liu,Y.C., Sachs,B.D. & Salamone,J.D. (1998) Sexual behavior in male rats after radiofrequency or dopamine-depleting lesions in nucleus accumbens. *Pharmacology Biochemistry and Behavior*, 60, 585-592.

Lloyd,S.A.C. & Dixson,A.F. (1988) Effects of Hypothalamic-Lesions Upon the Sexual and Social-Behavior of the Male Common Marmoset (Callithrix-Jacchus). *Brain Research*, 463, 317-329.

Lu,W., Jiang,W., Chen,J., Yin,M., Wang,Z. & Jiang,X. (2008) Modulation of brain delivery and copulation by intranasal apomorphine hydrochloride. *Int.J.Pharm.*, 349, 196-205.

Ludwig,M. (1998) Dendritic release of vasopressin and oxytocin. *J.Neuroendocrinol.*, 10, 881-895.

Ludwig,M., Sabatier,N., Dayanithi,G., Russell,J.A. & Leng,G. (2002a) The active role of dendrites in the regulation of magnocellular neurosecretory cell behavior. *Prog.Brain Res.*, 139, 247-256.

Ludwig,M., Sabatier,N., Bull,P.M., Landgraf, R., Dayanithi,G. & Leng,G. (2002b) Intracellular calcium stores regulate activity-dependent neuropeptide release from dendrites. *Nature.* 418(6893):85-9.

Ludwig,M. & Leng,G. (2006) Dendritic peptide release and peptide-dependent behaviours. *Nat Rev Neurosci.* 7(2):126-36.

Lumley,L.A. & Hull,E.M. (1999) Effects of a D1 antagonist and of sexual experience on copulation-induced Fos-like immunoreactivity in the medial preoptic nucleus. *Brain Res.*, 829, 55-68.

Mack,S.O., Kc,P., Wu,M., Coleman,B.R., Tolentino-Silva,F.P. & Haxhiu,M.A. (2002) Paraventricular oxytocin neurons are involved in neural modulation of breathing. *Journal of Applied Physiology*, 92, 826-834.

MacLennan,A.J., Lee,N., Vincent,S.R. & Walker,D.W. (1994) D2 dopamine receptor mRNA distribution in cholinergic and somatostatinergic cells of the rat caudate-putamen and nucleus accumbens. *Neurosci.Lett.*, 180, 214-218.

Maeda,N., Matsuoka,N. & Yamaguchi,I. (1994) Role of the dopaminergic, serotonergic and cholinergic link in the expression of penile erection in rats. *Jpn.J.Pharmacol.*, 66, 59-66.

Maillard,C.A. & Edwards,D.A. (1991) Excitotoxin lesions of the zona incerta/lateral tegmentum continuum: effects on male sexual behavior in rats. *Behav.Brain Res.*, 46, 143-149.

Maltais,S., te,C., Drolet,G. & Falardeau,P. (2000) Cellular colocalization of dopamine D1 mRNA and D2 receptor in rat brain using a D2 dopamine receptor specific polyclonal antibody. *Prog.Neuropsychopharmacol.Biol.Psychiatry*, 24, 1127-1149.

Mansour,A., Meador-Woodruff,J.H., Bunzow,J.R., Civelli,O., Akil,H. & Watson,S.J. (1990) Localization of dopamine D2 receptor mRNA and D1 and D2 receptor binding in the rat brain and pituitary: an in situ hybridization-receptor autoradiographic analysis. *J.Neurosci.*, 10, 2587-2600.

Manzo,J., Cruz,M.R., Hernandez,M.E., Pacheco,P. & Sachs,B.D. (1999) Regulation of noncontact erection in rats by gonadal steroids. *Horm.Behav.*, 35, 264-270.

Markowski,V.P., Eaton,R.C., Lumley,L.A., Moses,J. & Hull,E.M. (1994) A D-1 Agonist in the Mpoa Facilitates Copulation in Male-Rats. *Pharmacology Biochemistry and Behavior*, 47, 483-486.

Marson,L., List,M.S. & McKenna,K.E. (1992) Lesions of the nucleus paragigantocellularis alter ex copula penile reflexes. *Brain Res.*, 592, 187-192.

Marson,L. & McKenna,K.E. (1992) A role for 5-hydroxytryptamine in descending inhibition of spinal sexual reflexes. *Exp.Brain Res.*, 88, 313-320.

Martin,W.J., McGowan,E., Cashen,D.E., Gantert,L.T., Drisko,J.E., Hom,G.J., Nargund,R., Sebhat,I., Howard,A.D., Van der Ploeg,L.H. & MacIntyre,D.E. (2002) Activation of melanocortin MC(4) receptors increases erectile activity in rats ex copula. *Eur.J.Pharmacol.*, 454, 71-79.

Martin,W.J. & MacIntyre,D.E. (2004) Melanocortin receptors and erectile function. *European Urology*, 45, 706-713.

Martinez-Pineiro,L., Brock,G., Trigo-Rocha,F., Hsu,G.L., Lue,T.F. & Tanagho,E.A. (1994) Rat model for the study of penile erection: pharmacologic and electrical-stimulation parameters. *Eur.Urol.*, 25, 62-70.

Martino,B., Hsieh,G.C., Hollingsworth,P.R., Mikusa,J.P., Moreland,R.B. & Bitner,R.S. (2005) Central oxytocinergic and dopaminergic mechanisms regulating penile erection in conscious rats. *Pharmacology Biochemistry and Behavior*, 81, 797-804.

Mason,W.T. (1983) Excitation by dopamine of putative oxytocinergic neurones in the rat supraoptic nucleus in vitro: evidence for two classes of continuously firing neurones. *Brain Res.*, 267, 113-121.

Matsumoto,K., Banno,Y., Murate,T., Akao,Y. & Nozawa,Y. (2005) Localization of sphingosine kinase-1 in mouse sperm acrosomes. *J.Histochem.Cytochem.*, 53, 243-247.

Matuszewich,L., Lorrain,D.S. & Hull,E.M. (2000) Dopamine release in the medial preoptic area of female rats in response to hormonal manipulation and sexual activity. *Behav Neurosci.* 114(4):772-82.

McCarthy,M.M., McDonald,C.H., Brooks,P.J. & Goldman,D. (1996) An anxiolytic action of oxytocin is enhanced by estrogen in the mouse. *Physiol Behav.* 60(5):1209-15.

McKenna,K.E., Chung,S.K. & McVary,K.T. (1991) A model for the study of sexual function in anesthetized male and female rats. *Am.J.Physiol*, 261, R1276-R1285.

McKenna,K.E. (1999) Central nervous system pathways involved in the control of penile erection. *Annu.Rev.Sex Res.*, 10, 157-183.

Meddle,S.L., Bishop,V.R., Gkoumassi,E., van Leeuwen,F.W. & Douglas,A.J. (2007) Dynamic changes in oxytocin receptor expression and activation at parturition in the rat brain. *Endocrinology.*, 148(10):5095-104.

Meisel,R.L., Camp,D.M. & Robinson,T.E. (1993) A microdialysis study of ventral striatal dopamine during sexual behavior in female Syrian hamsters. *Behav Brain Res.* 55(2):151-7.

Melis,M.R, Argiolas,A. & Gessa,G.L. (1986) Oxytocin-induced penile erection and yawning: site of action in the brain. *Brain Res.* 398(2):259-65.

Melis,M.R., Argiolas,A. & Gessa,G.L. (1987) Apomorphine-Induced Penile Erection and Yawning - Site of Action in Brain. *Brain Research*, 415, 98-104.

Melis,M.R., Argiolas,A. & Gessa,G.L. (1989) Evidence That Apomorphine Induces Penile Erection and Yawning by Releasing Oxytocin in the Central Nervous-System. *European Journal of Pharmacology*, 164, 565-570.

Melis,M.R., Argiolas,A. & Gessa,G.L. (1989) Apomorphine Increases Plasma Oxytocin Concentration in Male-Rats. *Neuroscience Letters*, 98, 351-355.

Melis,M.R., Argiolas,A., Stancampiano,R. & Gessa,G.L. (1990) Effect of apomorphine on oxytocin concentrations in different brain areas and plasma of male rats. *Eur.J.Pharmacol.*, 182, 101-107.

Melis,M.R., Stancampiano,R. & Argiolas,A. (1992) Hippocampal oxytocin mediates apomorphine-induced penile erection and yawning. *Pharmacol.Biochem.Behav.*, 42, 61-66.

Melis,M.R. & Argiolas,A. (1993) Nitric oxide synthase inhibitors prevent apomorphine- and oxytocin-induced penile erection and yawning in male rats. *Brain Res.Bull.*, 32, 71-74.

Melis,M.R., Stancampiano,R. & Argiolas,A. (1994) Penile erection and yawning induced by paraventricular NMDA injection in male rats are mediated by oxytocin. *Pharmacol.Biochem.Behav.*, 48, 203-207.

Melis,M.R., Stancampiano,R. & Argiolas,A. (1994) Prevention by NG-nitro-L-arginine methyl ester of apomorphine- and oxytocin-induced penile erection and yawning: site of action in the brain. *Pharmacol.Biochem.Behav.*, 48, 799-804.

Melis,M.R. & Argiolas,A. (1995) Nitric oxide donors induce penile erection and yawning when injected in the central nervous system of male rats. *Eur.J.Pharmacol.*, 294, 1-9.

Melis,M.R., Succu,S., Iannucci,U. & Argiolas,A. (1997) Oxytocin increases nitric oxide production in the paraventricular nucleus of the hypothalamus of male rats: correlation with penile erection and yawning. *Regul.Pept.*, 69, 105-111.

Melis,M.R., Spano,M.S., Succu,S. & Argiolas,A. (1999) The oxytocin antagonist d(CH2)(5)Tyr(Me)(2)-Orn(8)-vasotocin reduces non-contact penile erections in male rats. *Neuroscience Letters*, 265, 171-174.

Melis,M.R., Succu,S., Spano,M.S. & Argiolas,A. (2000) Effect of excitatory amino acid, dopamine, and oxytocin receptor antagonists on noncontact penile erections and paraventricular nitric oxide production in male rats. *Behav.Neurosci.*, 114, 849-857.

Melis,M.R. & Argiolas,A. (2002) Reduction of drug-induced yawning and penile erection and of noncontact erections in male rats by the activation of GABAA receptors in the paraventricular nucleus: involvement of nitric oxide. *Eur.J.Neurosci.*, 15, 852-860.

Melis,M.R., Succu,S., Mascia,M.S., Cortis,L. & Argiolas,A. (2003) Extra-cellular dopamine increases in the paraventricular nucleus of male rats during sexual activity. *Eur.J.Neurosci.*, 17, 1266-1272.

Melis,M.R. & Argiolas,A. (2003) Central oxytocinergic neurotransmission: A drug target for the therapy of psychogenic erectile dysfunction. *Current Drug Targets*, 4, 55-66.

Melis,M.R., Succu,S., Mascia,M.S., Cortis,L. & Argiolas,A. (2004) Extracellular excitatory amino acids increase in the paraventricular nucleus of male rats during sexual activity: main role of N-methyl-d-aspartic acid receptors in erectile function. *Eur.J.Neurosci.*, 19, 2569-2575.

Melis,M.R., Succu,S., Mascia,M.S. & Argiolas,A. (2005) PD-168077, a selective dopamine D-4 receptor agonist, induces penile erection when injected into the paraventricular nucleus of male rats. *Neuroscience Letters*, 379, 59-62.

Melis,M.R., Succu,S., Mascia,M.S., Sanna,F., Melis,T., Castelli,M.P. & Argiolas,A. (2006) The cannabinoid receptor antagonist SR-141716A induces penile erection in male rats: involvement of paraventricular glutamic acid and nitric oxide. *Neuropharmacology*, 50, 219-228.

Melis,M.R., Succu,S., Sanna,F., Melis,T., Mascia,M.S., Enguehard-Gueiffier,C., Hubner,H., Gmeiner,P., Gueiffier,A. & Argiolas,A. (2006) PIP3EA and PD-168077, two selective dopamine D4 receptor agonists, induce penile erection in male rats: site and mechanism of action in the brain. *European Journal of Neuroscience*, 24, 2021-2030.

Melis,M.R., Melis,T., Cocco,C., Succu,S., Sanna,F., Pillolla,G., Boi,A., Ferri,G.L. & Argiolas,A. (2007) Oxytocin injected into the ventral tegmental area induces penile erection and increases extracellular dopamine in the nucleus accumbens and paraventricular nucleus of the hypothalamus of male rats. *Eur.J.Neurosci.*, 26, 1026-1035.

Mignini,F., Bronzetti,E., Felici,L., Ricci,A., Sabbatini,M., Tayebati,S.K. & Amenta,F. (2000) Dopamine receptor immunohistochemistry in the rat choroid plexus. *J.Auton.Pharmacol.*, 20, 325-332.

Millan,M.J., Dekeyne,A., Rivet,J.M., Dubuffet,T., Lavielle,G. & Brocco,M. (2000) S33084, a novel, potent, selective, and competitive antagonist at dopamine D(3)-receptors: II. Functional and behavioral profile compared with GR218,231 and L741,626. *J.Pharmacol.Exp.Ther.* , 293, 1063-1073.

Mills,T.M., Wiedmeier,V.T. & Stopper,V.S. (1992) Androgen maintenance of erectile function in the rat penis. *Biol.Reprod.*, 46, 342-348.

Mills,T.M. & Lewis,R.W. (1999) The Role of Andorgens in the Erectile Response: A 1999 Perspective. *Mol.Urol.*, 3, 75-86.

Miner,M.M. & Seftel,A.D. (2007) Centrally acting mechanisms for the treatment of male sexual dysfunction. *Urol.Clin.North Am.*, 34, 483-96.

Minsky,M (1988) Memoir on inventing confocal scanning microscope. *Scanning.* 10, 128-138

Missale,C., Nash,S.R., Robinson,S.W., Jaber,M. & Caron,M.G. (1998) Dopamine receptors: from structure to function. *Physiol Rev.* 78, 189-225.

Mizusawa,H., Hedlund,P. & Andersson,K.E. (2002) alpha-Melanocyte stimulating hormone and oxytocin induced penile erections, and intracavernous pressure increases in the rat. *J.Urol.*, 167, 757-760.

Monaghan,E.P., Arjomand,J. & Breedlove,S.M. (1993) Brain-Lesions Affect Penile Reflexes. *Hormones and Behavior*, 27, 122-131.

Mones,R.J., Elizan,T.S. & Siegel,G.J. (1970) Evaluation of L-dopa therapy in Parkinson's disease. *N.Y.State J.Med.*, 70, 2309-2318.

Mong,J.A. & McCarthy,M.M. (1999) Steroid-induced developmental plasticity in hypothalamic astrocytes: implications for synaptic patterning. *J.Neurobiol.*, 40, 602-619.

Moos,F, Freund-Mercier,M.J., Guerné,Y., Guerné,J.M., Stoeckel,M.E. & Richard,P. (1984) Release of oxytocin and vasopressin by magnocellular nuclei in vitro: specific facilitatory effect of oxytocin on its own release. *J Endocrinol.* 102(1):63-72.

Moos,F., Poulain,D.A., Rodriguez,F., Guerne,Y., Vincent,J.D. & Richard,P. (1989) Release of oxytocin within the supraoptic nucleus during the milk ejection reflex in rats. *Exp.Brain Res.*, 76, 593-602.

Moreland,R.B., Nakane,M., Donnelly-Roberts,D.L., Miller,L.N., Chang,R., Uchic,M.E., Terranova,M.A., Gubbins,E.J., Helfrich,R.J., Namovic,M.T., El-Kouhen,O.F., Masters,J.N. &

Brioni,J.D. (2004) Comparative pharmacology of human dopamine D(2)-like receptor stable cell lines coupled to calcium flux through Galpha(qo5). *Biochem.Pharmacol.*, 68, 761-772.

Moreland,R.B., Patel,M., Hsieh,G.C., Wetter,J.M., Marsh,K. & Brioni,J.D. (2005) A-412997 is a selective doparnine D-4 receptor agonist in rats. *Pharmacology Biochemistry and Behavior*, 82, 140-147.

Morris,K. (2003) Melanocortins key to trigger sex on the brain. *Lancet Neurol.*, 2, 140.

Moses,J., Loucks,J.A., Watson,H.L., Matuszewich,L. & Hull,E.M. (1995) Dopaminergic drugs in the medial preoptic area and nucleus accumbens: effects on motor activity, sexual motivation, and sexual performance. *Pharmacol.Biochem.Behav.*, 51, 681-686.

Mrzljak,L., Bergson,C., Pappy,M., Huff,R., Levenson,R. & Goldman-Rakic,P.S. (1996) Localization of dopamine D4 receptors in GABAergic neurons of the primate brain. *Nature*, 381, 245-248.

Murphy,M.R., Seckl,J.R., Burton,S., Checkley,S.A. & Lightman,S.L. (1987) Changes in oxytocin and vasopressin secretion during sexual activity in men. *J.Clin.Endocrinol.Metab*, 65, 738-741.

Muschamp,J.W., Dominguez,J.M., Sato,S.M., Shen,R.Y. & Hull,E.M. (2007) A role for hypocretin (orexin) in male sexual behavior. *J.Neurosci.*, 27, 2837-2845.

Nadelhaft,I. & Booth,A.M. (1984) The location and morphology of preganglionic neurons and the distribution of visceral afferents from the rat pelvic nerve: a horseradish peroxidase study. *J.Comp Neurol.*, 226, 238-245.

Nadelhaft,I. & McKenna,K.E. (1987) Sexual dimorphism in sympathetic preganglionic neurons of the rat hypogastric nerve. *J.Comp Neurol.*, 256, 308-315.

Neumann,I., Russell,J.A. & Landgraf,R. (1993a) Oxytocin and vasopressin release within the supraoptic and paraventricular nuclei of pregnant, parturient and lactating rats: a microdialysis study. *Neuroscience*, 53, 65-75.

Neumann, I,. Ludwig, M., Engelmann, M., Pittman, Q.J. & Landgraf, R. (1993b) Simultaneous microdialysis in blood and brain: oxytocin and vasopressin release in response to central and peripheral osmotic stimulation and suckling in the rat. *Neuroendocrinology.* 1993 Dec;58(6):637-45.

Neumann,I.D., Toschi,N., Ohl,F., Torner,L. & Kromer,S.A. (2001b) Maternal defence as an emotional stressor in female rats: correlation of neuroendocrine and behavioural parameters and involvement of brain oxytocin. *European Journal of Neuroscience*, 13, 1016-1024.

Neumann ,I.D. (2001a) Alterations in behavioral and neuroendocrine stress coping strategies in pregnant, parturient and lactating rats. *Prog Brain Res.* 2001;133:143-52. Review.

Nguyen,T.V., Kosofsky,B.E., Birnbaum,R., Cohen,B.M. & Hyman,S.E. (1992) Differential expression of c-fos and zif268 in rat striatum after haloperidol, clozapine, and amphetamine. *Proc.Natl.Acad.Sci.U.S.A*, 89, 4270-4274.

Nicholson,H.D. & Jenkin,L. (1995) Oxytocin and prostatic function. *Adv Exp Med Biol.* 395:529-38.

Nishioka,T., nselmo-Franci,J.A., Li,P., Callahan,M.F. & Morris,M. (1998) Stress increases oxytocin release within the hypothalamic paraventricular nucleus. *Brain Res.*, 781, 56-60.

O'Sullivan,J.D. & Hughes,A.J. (1998) Apomorphine-induced penile erections in Parkinson's disease. *Mov Disord.* , 13, 536-539.

Ogier,R., Tribollet,E., Suarez,P. & Raggenbass,M. (2006) Identified motoneurons involved in sexual and eliminative functions in the rat are powerfully excited by vasopressin and tachykinins. *J.Neurosci.*, 26, 10717-10726.

Papatsoris,A.G., Deliveliotis,C., Singer,C. & Papapetropoulos,S. (2006) Erectile dysfunction in Parkinson's disease. *Urology*, 67, 447-451.

Paredes,R.G. (2003) Medial preoptic area/anterior hypothalamus and sexual motivation. *Scandinavian Journal of Psychology*, 44, 203-212.

Paredes,R.G. & Agmo,A. (2004) Has dopamine a physiological role in the control of sexual behavior? A critical review of the evidence. *Progress in Neurobiology*, 73, 179-226.

Parfitt,D.B. & Newman,S.W. (1998) Fos-immunoreactivity within the extended amygdala is correlated with the onset of sexual satiety. *Horm.Behav.*, 34, 17-29.

Pattij,T., de Jong,T.R., Uitterdijk,A., Waldinger,M.D., Veening,J.G., Cools,A.R., van der Graaf,P.H. & Olivier,B. (2005) Individual differences in male rat ejaculatory behaviour: searching for models to study ejaculation disorders. *European Journal of Neuroscience*, 22, 724-734.

Pedersen,C.A., Caldwell,J.D., Peterson,G., Walker,C.H. & Mason,G.A. (1992) Oxytocin Activation of Maternal-Behavior in the Rat. *Annals of the New York Academy of Sciences*, 652, 58-69.

Pedersen,C.A. & Boccia,M.L. (2006) Vasopressin interactions with oxytocin in the control of female sexual behavior. *Neuroscience*, 139, 843-851.

Pehek,E.A., Thompson,J.T., Eaton,R.C., Bazzett,T.J. & Hull,E.M. (1988) Apomorphine and Haloperidol, But Not Domperidone, Affect Penile Reflexes in Rats. *Pharmacology Biochemistry and Behavior*, 31, 201-208.

Pescatori,E.S., Calabro,A., Artibani,W., Pagano,F., Triban,C. & Italiano,G. (1993) Electrical stimulation of the dorsal nerve of the penis evokes reflex tonic erections of the penile body and reflex ejaculatory responses in the spinal rat. *J.Urol.*, 149, 627-632.

Petersson, M. (2002) Cardiovascular effects of oxytocin. *Prog Brain Res.* 139:281-8.

Petrov,T., Krukoff,T.L. & Jhamandas,J.H. (1995) Convergent influence of the central nucleus of the amygdala and the paraventricular hypothalamic nucleus upon brainstem autonomic neurons as revealed by c-fos expression and anatomical tracing. *Journal of Neuroscience Research*, 42, 835-845.

Pettibone,D.J., Guidotti,M., Harrell,C.M., Jasper,J.R., Lis,E.V., O'Brien,J.A., Reiss,D.R., Woyden,C.J., Bock,M.G., Evans,B.E. & . (1995) Progress in the development of oxytocin antagonists for use in preterm labor. *Adv.Exp.Med.Biol.*, 395, 601-612.

Pfaus,J.G. & Phillips,A.G. (1989) Differential effects of dopamine receptor antagonists on the sexual behavior of male rats. *Psychopharmacology (Berl)*, 98, 363-368.

Pfaus,J.G., Damsma,G., Nomikos,G.G., Wenkstern,D.G., Blaha,C.D., Phillips,A.G. & Fibiger,H.C. (1990) Sexual behavior enhances central dopamine transmission in the male rat. *Brain Res.*, 530, 345-348.

Pfaus,J.G. & Phillips,A.G. (1991) Role of dopamine in anticipatory and consummatory aspects of sexual behavior inthe male rat. *Behav Neurosci.*, 105(5):727-43.

Pfaus,J.G., Damsma,G., Wenkstern,D. & Fibiger,H.C. (1995) Sexual activity increases dopamine transmission in the nucleus accumbens and striatum of female rats. *Brain Res.* 693(1-2):21-30.

Pfaus,J.G. & Heeb,M.M. (1997) Implications of immediate-early gene induction in the brain following sexual stimulation of female and male rodents. *Brain Research Bulletin*, 44, 397-407.

Phillips-Farfan,B.V. & Fernandez-Guasti,A. (2007) c-Fos expression related to sexual satiety in the male rat forebrain. *Physiology & Behavior*, 91, 609-619.

Pinto,A., Jankowski,M. & Sesack,S.R. (2003) Projections from the paraventricular nucleus of the thalamus to the rat prefrontal cortex and nucleus accumbens shell: ultrastructural characteristics and spatial relationships with dopamine afferents. *J.Comp Neurol.*, 459, 142-155.

Polak, J.M. (2003) *Introduction to immunocytochemistry*, 3rd Ed. 5-90

Pomerantz,S.M. (1991) Quinelorane (LY163502), a D2 dopamine receptor agonist, acts centrally to facilitate penile erections of male rhesus monkeys. *Pharmacol.Biochem.Behav.*, 39, 123-128.

Portillo W, Díaz NF, Cabrera EA, Fernández-Guasti A, Paredes RG. (2006) Comparative analysis of immunoreactive cells for androgen receptors and oestrogen receptor alpha in copulating and non-copulating male rats. *J Neuroendocrinol.* 18(3):168-76.

Prewitt,C.M. & Herman,J.P. (1998) Anatomical interactions between the central amygdaloid nucleus and the hypothalamic paraventricular nucleus of the rat: a dual tract-tracing analysis. *J.Chem.Neuroanat.*, 15, 173-185.

Price,C.J. & Pittman,Q.J. (2001) Dopamine D4 receptor activation inhibits presynaptically glutamatergic neurotransmission in the rat supraoptic nucleus. *J.Neurophysiol.*, 86, 1149-1155.

Primus,R.J., Thurkauf,A., Xu,J., Yevich,E., McInerney,S., Shaw,K., Tallman,J.F. & Gallagher,D.W. (1997) II. Localization and characterization of dopamine D4 binding sites in rat and human brain by use of the novel, D4 receptor-selective ligand [3H]NGD 94-1. *J.Pharmacol.Exp.Ther.*, 282, 1020-1027.

Purohit,R.C. & Beckett,S.D. (1976) Penile pressures and muscle activity associated with erection and ejaculation in the dog. *Am J Physiol.* 231(5 Pt. 1):1343-8.

Putnam,S.K., Du,J., Sato,S. & Hull,E.M. (2001) Testosterone restoration of copulatory behavior correlates with medial preoptic dopamine release in castrated male rats. *Horm.Behav.*, 39, 216-224.

Putnam,S.K., Sato,S., Riolo,J.V. & Hull,E.M. (2005) Effects of testosterone metabolites on copulation, medial preoptic dopamine, and NOS-immunoreactivity in castrated male rats. *Horm.Behav.* 47, 513-522.

Quysner,A. & Blaustein,J.D. (2001) A dopamine antagonist blocks vaginocervical stimulation-induced neuronal responses in the rat forebrain. *Brain Res.* 921(1-2):173-82.

Rajfer,J., Aronson,W.J., Bush,P.A., Dorey,F.J. & Ignarro,L.J. (1992) Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. *N.Engl.J.Med.*, 326, 90-94.

Ralph,D.J. (2005) Normal erectile function. *Clin.Cornerstone.*, 7, 13-18.

Rampin,O., Monnerie,R., Jerome,N., McKenna,K. & Maurin,Y. (2004) Spinal control of erection by glutamate in rats. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, 286, R710-R718.

Renaud,L.P. & Bourque,C.W. (1991) Neurophysiology and neuropharmacology of hypothalamic magnocellular neurons secreting vasopressin and oxytocin. *Prog.Neurobiol.*, 36, 131-169.

Richard,P., Moos,F. & Freundmercier,M.J. (1991) Central Effects of Oxytocin. *Physiological Reviews*, 71, 331-370.

Richard,P., Moos,F., Dayanithi,G., Gouzenes,L. & Sabatier,N. (1997) Rhythmic activities of hypothalamic magnocellular neurons: autocontrol mechanisms. *Biol.Cell*, 89, 555-560.

Ring,R.H., Malberg,J.E., Potestio,L., Ping,J., Boikess,S., Luo,B., Schechter,L.E., Rizzo,S., Rahman, Z. & Rosenzweig-Lipson,S. (2006) Anxiolytic-like activity of oxytocin in male mice: behavioral and autonomic evidence, therapeutic implications. *Psychopharmacology (Berl).*185(2):218-25

Rivkees,S.A. & Lachowicz,J.E. (1997) Functional D1 and D5 dopamine receptors are expressed in the suprachiasmatic, supraoptic, and paraventricular nuclei of primates. *Synapse*, 26, 1-10.

Robertson,G.S., Pfaus,J.G., Atkinson,L.J., Matsumura,H., Phillips,A.G. & Fibiger,H.C. (1991) Sexual-Behavior Increases C-Fos Expression in the Forebrain of the Male-Rat. *Brain Research*, 564, 352-357.

Robertson,H.A. (1992) Dopamine receptor interactions: some implications for the treatment of Parkinson's disease. *Trends Neurosci.*, 15, 201-206.

Robertson,H.A. (1992) Synergistic interactions of D1- and D2-selective dopamine agonists in animal models for Parkinson's disease: sites of action and implications for the pathogenesis of dyskinesias. *Can.J.Neurol.Sci.*, 19, 147-152.

Robinson,B.W. & Mishkin,M. (1968) Penile Erection Evoked from Forebrain Structures in Macaca Mulatta. *Archives of Neurology*, 19, 184-&.

Roeling,T.A., Veening,J.G., Peters,J.P., Vermelis,M.E. & Nieuwenhuys,R. (1993) Efferent connections of the hypothalamic "grooming area" in the rat. *Neuroscience*, 56, 199-225.

Rogue,P. & Vincendon,G. (1992) Dopamine D2 receptor antagonists induce immediate early genes in the rat striatum. *Brain Res.Bull.*, 29, 469-472.

Romero,R., Sibai,B.M., Sanchez-Ramos,L., Valenzuela,G.J., Veille,J.C., Tabor,B., Perry,K.G., Varner,M., Goodwin,T.M., Lane,R., Smith,J., Shangold,G. & Creasy,G.W. (2000) An oxytocin

receptor antagonist (atosiban) in the treatment of preterm labor: a randomized, double-blind, placebo-controlled trial with tocolytic rescue. *Am.J.Obstet.Gynecol.*, 182, 1173-1183.

Russell JA, Douglas AJ, Ingram CD. (2001) Brain preparations for maternity--adaptive changes in behavioral and neuroendocrine systems during pregnancy and lactation. An overview. *Prog Brain Res.* 133:1-38.

Russell,J.A., Leng,G. & Douglas,A.J. (2003) The magnocellular oxytocin system, the fount of maternity: adaptations in pregnancy. *Frontiers in Neuroendocrinology*, 24, 27-61.

Ryan,K.J., Naftolin,F., Reddy,V., Flores,F. & Petro,Z. (1972) Estrogen formation in the brain. *Am J Obstet Gynecol.*114(4):454-60.

Sabatier,N., Caquineau,C., Dayanithi,G., Bull,P., Douglas,A.J., Guan,X.M.M., Jiang,M., Van der Ploeg,L. & Leng,G. (2003) alpha-melanocyte-stimulating hormone stimulates oxytocin release from the dendrites of hypothalamic neurons while inhibiting oxytocin release from their terminals in the neurohypophysis. *Journal of Neuroscience*, 23, 10351-10358.

Sachs,B.D. & Leipheimer,R.E. (1988) Rapid effect of testosterone on striated muscle activity in rats. *Neuroendocrinology.* 48(5):453-8.

Salamone,J.D. (1992) Complex Motor and Sensorimotor Functions of Striatal and Accumbens Dopamine - Involvement in Instrumental Behavior Processes. *Psychopharmacology*, 107, 160-174.

Sanghera,M.K., Anselmofranci,J. & Mccann,S.M. (1991) Effect of Medial Zona Incerta Lesions on the Ovulatory Surge of Gonadotropins and Prolactin in the Rat. *Neuroendocrinology*, 54, 433-438.

Sansone,G.R., Gerdes,C.A., Steinman,J.L., Winslow,J.T., Ottenweller,J.E., Komisaruk,B.R. & Insel,T.R. (2002) Vaginocervical stimulation releases oxytocin within the spinal cord in rats. *Neuroendocrinology*, 75, 306-315.

Sato,S., Braham,C.S., Putnam,S.K. & Hull,E.M. (2005) Neuronal nitric oxide synthase and gonadal steroid interaction in the MPOA of male rats: co-localization and testosterone-induced restoration of copulation and nNOS-immunoreactivity. *Brain Res.* 1043(1-2):205-13.

Sato,S.M. & Hull,E.M. (2006) The nitric oxide-guanosine 3',5'-cyclic monophosphate pathway regulates dopamine efflux in the medial preoptic area and copulation in male rats. *Neuroscience*, 139, 417-428.

Sato,Y. & Christ,G.J. (2000) Differential ICP responses elicited by electrical stimulation of medial preoptic area. *American Journal of Physiology-Heart and Circulatory Physiology*, 278, H964-H970.

Sawchenko,P.E. & Swanson,L.W. (1985) Localization, colocalization, and plasticity of corticotropin-releasing factor immunoreactivity in rat brain. *Fed Proc.* 44(1 Pt 2):221-7.

Scaletta,L.L. & Hull,E.M. (1990) Systemic or intracranial apomorphine increases copulation in long-term castrated male rats. *Pharmacol.Biochem.Behav.*, 37, 471-475.

Schmidt,M.H., Valatx,J.L., Sakai,K., Debilly,G. & Jouvet,M. (1995) Corpus spongiosum penis pressure and perineal muscle activity during reflexive erections in the rat. *Am J Physiol.* 269(4 Pt 2):R904-13.

Schulze,H.G. & Gorzalka,B.B. (1991) Oxytocin Effects on Lordosis Frequency and Lordosis Duration Following Infusion Into the Medial Pre-Optic Area and Ventromedial Hypothalamus of Female Rats. *Neuropeptides*, 18, 99-106.

Serradeil-Le,G.C., Valette,G., Foulon,L., Germain,G., Advenier,C., Naline,E., Bardou,M., Martinolle,J.P., Pouzet,B., Raufaste,D., Garcia,C., Double-Cazanave,E., Pauly,M., Pascal,M., Barbier,A., Scatton,B., Maffrand,J.P. & Le,F.G. (2004) SSR126768A (4-chloro-3-[(3R)-(+)-5-chloro-1-(2,4-dimethoxybenzyl)-3-methyl-2-oxo-2,3-dihydro -1H-indol-3-yl]-N-ethyl-N-(3-pyridylmethyl)-benzamide, hydrochloride): a new selective and orally active oxytocin receptor antagonist for the prevention of preterm labor. *J.Pharmacol.Exp.Ther.*, 309, 414-424.

Sezen,S.F. & Burnett,A.L. (2000) Intracavernosal pressure monitoring in mice: responses to electrical stimulation of the cavernous nerve and to intracavernosal drug administration. *J.Androl* , 21, 311-315.

Shafer,R.A. & Levant,B. (1998) The D3 dopamine receptor in cellular and organismal function. *Psychopharmacology (Berl)*, 135, 1-16.

Shapiro,S.K. (1973) Hypersexual behavior complicating levodopa (I-dopa) therapy. *Minn.Med.*, 56, 58-59.

Shimura,T., Yamamoto,T. & Shimokochi,M. (1994) The Medial Preoptic Area Is Involved in Both Sexual Arousal and Performance in Male-Rats - Reevaluation of Neuron Activity in Freely Moving Animals. *Brain Research*, 640, 215-222.

Shyken,J.M. & Petrie,R.H. (1995) The use of oxytocin. *Clin.Perinatol.*, 22, 907-931.

Shyken,J.M. & Petrie,R.H. (1995) Oxytocin to induce labor. *Clin.Obstet.Gynecol.*, 38 , 232-245.

Smeltzer MD, Curtis JT, Aragona BJ, Wang Z. (2006) Dopamine, oxytocin, and vasopressin receptor binding in the medial prefrontal cortex of monogamous and promiscuous voles. *Neurosci Lett.* 394(2):146-51.

Smith,J.G. & Merrill,D.C. (2006) Oxytocin for induction of labor. *Clin.Obstet.Gynecol.*, 49, 594-608.

Sofroniew,M.V. & Weindl,A. (1978) Extrahypothalamic neurophysin-containing perikarya, fiber pathways and fiber clusters in the rat brain. *Endocrinology.* 102(1):334-7.

Sokoloff,P. & Schwartz,J.C. (1995) Novel dopamine receptors half a decade later. *Trends Pharmacol.Sci.*, 16, 270-275.

Sperling,H., Lummen,G., Schneider,T. & Rubben,H. (2003) [New treatment options for erectile dysfunction. Pharmacologic and nonpharmacologic options]. *Herz*, 28, 314-324.

Stafford,S.A., Tang,K. & Coote,J.H. (2006) Activation of lumbosacral 5-HT2C receptors induces bursts of rhythmic activity in sympathetic nerves to the vas deferens in male rats. *British Journal of Pharmacology*, 148, 1083-1090.

Stancampiano,R., Melis,M.R. & Argiolas,A. (1994) Penile erection and yawning induced by 5-HT1C receptor agonists in male rats: relationship with dopaminergic and oxytocinergic transmission. *Eur.J.Pharmacol.*, 261, 149-155.

Steers,W.D., Albo,M. & Vanasselt,E. (1992) Effects of Serotonergic Agonists on Micturition and Sexual Function in the Rat. *Drug Development Research*, 27, 361-375.

Stoneham,M.D., Everitt,B.J., Hansen,S., Lightman,S.L. & Todd,K. (1985) Oxytocin and sexual behaviour in the male rat and rabbit. *J.Endocrinol.*, 107, 97-106.

Struthers,W.M. (2001) Sex-induced Fos in the medial preoptic area: projections to the midbrain. *Neuroreport*, 12, 3065-3068.

Succu,S., Spano,M.S., Melis,M.R. & Argiolas,A. (1998) Different effects of omega-conotoxin on penile erection, yawning and paraventricular nitric oxide in male rats. *Eur.J.Pharmacol.*, 359, 19-26.

Succu,S., Mascia,M.S., Sanna,F., Melis,T., Argiolas,A. & Melis,M.R. (2006) The cannabinoid CB1 receptor antagonist SR 141716A induces penile erection by increasing extra-cellular glutamic acid in the paraventricular nucleus of male rats. *Behav.Brain Res.*, 169, 274-281.

Succu,S., Sanna,F., Melis,T., Boi,A., Argiolas,A. & Melis,M.R. (2007) Stimulation of dopamine receptors in the paraventricular nucleus of the hypothalamus of male rats induces penile erection and increases extra-cellular dopamine in the nucleus accumbens: Involvement of central oxytocin. *Neuropharmacology*, 52, 1034-1043.

Surmeier,D.J., Song,W.J. & Yan,Z. (1996) Coordinated expression of dopamine receptors in neostriatal medium spiny neurons. *J.Neurosci.*, 16, 6579-6591.

Suzuki,N., Sato,Y., Hisasue,S., Kato,R., Suzuki,K. & Tsukamoto,T. (2007) Effect of testosterone on intracavernous pressure elicited with electrical stimulation of the medial preoptic area and cavernous nerve in male rats. *J.Androl*, 28, 218-222.

Swanson,L.W. & Mckellar,S. (1979) Distribution of Oxytocin-Stained and Neurophysin-Stained Fibers in the Spinal-Cord of the Rat and Monkey. *Journal of Comparative Neurology*, 188, 87-106.

Swanson,L.W., Sawchenko,P.E., Wiegand,S.J. & Price,J.L. (1980) Separate neurons in the paraventricular nucleus project to the median eminence and to the medulla or spinal cord. *Brain Res.*, 198, 190-195.

Swanson,L.W. & Sawchenko,P.E. (1980) Paraventricular nucleus: a site for the integration of neuroendocrine and autonomic mechanisms. *Neuroendocrinology*, 31, 410-417.

Swanson,L.W. & Kuypers,H.G.J.M. (1980) The Paraventricular Nucleus of the Hypothalamus - Cytoarchitectonic Subdivisions and Organization of Projections to the Pituitary, Dorsal Vagal Complex, and Spinal-Cord As Demonstrated by Retrograde Fluorescence Double-Labeling Methods. *Journal of Comparative Neurology*, 194, 555-&.

Takeuchi,Y. & Fukunaga,K. (2003) Differential subcellular localization of two dopamine D2 receptor isoforms in transfected NG108-15 cells. *J.Neurochem.*, 85, 1064-1074.

Tang,Y., Rampin,O., Calas,A., Facchinetti,P. & Giuliano,F. (1998) Oxytocinergic and serotonergic innervation of identified lumbosacral nuclei controlling penile erection in the male rat. *Neuroscience*, 82, 241-254.

Tang,Y., Rampin,O., Giuliano,F. & Ugolini,G. (1999) Spinal and brain circuits to motoneurons of the bulbospongiosus muscle: Retrograde transneuronal tracing with rabies virus. *Journal of Comparative Neurology*, 414, 167-192.

Tanner,K.M., Obasi,C., Herrick,I.A. & Leung,L.S. (2000) Effects of propofol on hippocampal synaptic transmission in behaving rats. *Anesthesiology*, 93, 463-472.

Temel,Y., Hafizi,S., Tan,S. & Visser-Vandewalle,V. (2006) Role of the brain in the control of erection. *Asian J.Androl*, 8, 259-264.

Terenzi,M.G. & Ingram,C.D. (2005) Oxytocin-induced excitation of neurones in the rat central and medial amygdaloid nuclei. *Neuroscience.* 134(1):345-54.

Toth,Z.E., Gallatz,K., Fodor,M. & Palkovits,M. (1999) Decussations of the descending paraventricular pathways to the brainstem and spinal cord autonomic centers. *Journal of Comparative Neurology*, 414, 255-266.

Traish,A.M. & Guay,A.T. (2006) Are androgens critical for penile erections in humans? Examining the clinical and preclinical evidence. *J.Sex Med.*, 3, 382-404.

Truitt,W.A. & Coolen,L.M. (2002) Identification of a potential ejaculation generator in the spinal cord. *Science*, 297, 1566-1569.

Uckert,S., Becker,A.J., Ness,B.O., Stief,C.G., Scheller,F., Knapp,W.H. & Jonas,U. (2003) Oxytocin plasma levels in the systemic and cavernous blood of healthy males during different penile conditions. *World Journal of Urology*, 20, 323-326.

Uckert,S., Becker,A.J., Ness,B.O., Stief,C.G., Scheller,F., Knapp,W.H. & Jonas,U. (2003) Oxytocin plasma levels in the systemic and cavernous blood of healthy males during different penile conditions. *World Journal of Urology*, 20, 323-326.

Urano A, Kobayashi H. (1978) Effects of noradrenaline and dopamine injected into the supraoptic nucleus on urine flow rate in hydrated rats. *Exp Neurol.* 60(1):140-50

Uvnas-Moberg,K., Alster,P., Hillegaart,V. & Ahlenius,S. (1995) Suggestive evidence for a DA D3 receptor-mediated increase in the release of oxytocin in the male rat. *Neuroreport*, 6, 1338-1340.

Vagell ME, McGinnis MY. (1997) The role of aromatization in the restoration of male rat reproductive behavior. *J Neuroendocrinol.* 9(6):415-21.

van den Pol,A.N. (1991) Glutamate and aspartate immunoreactivity in hypothalamic presynaptic axons. *J Neurosci.*, 11(7):2087-101.

Vargas,V.M., Torres,D., Corona,F., Vergara,M., Gomez,L.E., gado-Lezama,R. & Cueva-Rolon,R. (2004) Cholinergic facilitation of erection and ejaculation in spinal cord-transected rats. *Int.J.Impot.Res.*, 16, 86-90.

Veening,J.G. & Coolen,L.M. (1998) Neural activation following sexual behavior in the male and female rat brain. *Behavioural Brain Research*, 92, 181-193.

Vernaleken,I., Siessmeier,T., Buchholz,H.G., Hartter,S., Hiemke,C., Stoeter,P., Rosch,F., Bartenstein,P. & Grunder,G. (2004) High striatal occupancy of D2-like dopamine receptors by amisulpride in the brain of patients with schizophrenia. *Int.J.Neuropsychopharmacol.*, 7, 421-430.

Veronneau-Longueville,F., Rampin,O., Freund-Mercier,M.J., Tang,Y., Calas,A., Marson,L., McKenna,K.E., Stoeckel,M.E., Benoit,G. & Giuliano,F. (1999) Oxytocinergic innervation of autonomic nuclei controlling penile erection in the rat. *Neuroscience*, 93, 1437-1447.

Vignozzi,L., Filippi,S., Luconi,M., Morelli,A., Mancina,R., Marini,M., Vannelli,G.B., Granchi,S., Orlando,C., Gelmini,S., Ledda,F., Forti,G. & Maggi,M. (2004) Oxytocin receptor is expressed in the penis and mediates an estrogen-dependent smooth muscle contractility. *Endocrinology*. 145(4):1823-34.

Vincent,P.A. & Etgen,A.M. (1993) Steroid priming promotes oxytocin-induced norepinephrine release in the ventromedial hypothalamus of female rats. *Brain Res*. 620(2):189-94.

Wagner,C.K. & Clemens,L.G. (1991) Projections of the Paraventricular Nucleus of the Hypothalamus to the Sexually Dimorphic Lumbosacral Region of the Spinal-Cord. *Brain Research*, 539, 254-262.

Wagner,C.K., Sisk,C.L. & Clemens,L.G. (1993) Neurons in the paraventricular nucleus of the hypothalamus that project to the sexually dimorphic lower lumbar spinal cord concentrate 3H-estradiol in the male rat. *J.Neuroendocrinol.*, 5, 545-551.

Waldherr,M. & Neumann,I.D. (2007) Centrally released oxytocin mediates mating-induced anxiolysis in male rats. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 16681-16684.

Warner,R.K., Thompson,J.T., Markowski,V.P., Loucks,J.A., Bazzett,T.J., Eaton,R.C. & Hull,E.M. (1991) Microinjection of the dopamine antagonist cis-flupenthixol into the MPOA impairs copulation, penile reflexes and sexual motivation in male rats. *Brain Res.*, 540, 177-182.

Watkins,T.W. & Keast,J.R. (1999) Androgen-sensitive preganglionic neurons innervate the male rat pelvic ganglion. *Neuroscience*, 93, 1147-1157.

Wedzony,K., Chocyk,A., Mackowiak,M., Fijal,K. & Czyrak,A. (2000) Cortical localization of dopamine D4 receptors in the rat brain--immunocytochemical study. *J.Physiol Pharmacol.*, 51, 205-221.

Weil-Fugazza,J. & Godefroy,F. (1993) Dorsal and ventral dopaminergic innervation of the spinal cord: functional implications. *Brain Res.Bull.*, 30, 319-324.

Wenkstern,D., Pfaus,J.G. & Fibiger,H.C. (1993) Dopamine transmission increases in the nucleus accumbens of male rats during their first exposure to sexually receptive female rats. *Brain Res.*, 618, 41-46.

Wessells,H., Fuciarelli,K., Hansen,J., Hadley,M.E., Hruby,V.J., Dorr,R. & Levine,N. (1998) Synthetic melanotropic peptide initiates erections in men with psychogenic erectile dysfunction: double-blind, placebo controlled crossover study. *J.Urol.*, 160, 389-393.

Wessells,H., Hruby,V.J., Hackett,J., Han,G.X., Balse-Srinivasan,P. & Vanderah,T.W. (2003) MT-II induces penile erection via brain and spinal mechanisms. *Ann N Y Acad Sci,* 994, 90-95.

West,C.H.K., Clancy,A.N. & Michael,R.P. (1992) Enhanced Responses of Nucleus-Accumbens Neurons in Male-Rats to Novel Odors Associated with Sexually Receptive Females. *Brain Research*, 585, 49-55.

Whitman,D.C. & Albers,H.E. (1995) Role of Oxytocin in the Hypothalamic Regulation of Sexual Receptivity in Hamsters. *Brain Research*, 680, 73-79.

Wilke,R.A., Hsu,S.F. & Jackson,M.B. (1998) Dopamine D4 receptor mediated inhibition of potassium current in neurohypophysial nerve terminals. *J.Pharmacol.Exp.Ther.*, 284, 542-548.

Wilson CA, Davies DC. (2007) The control of sexual differentiation of the reproductive system and brain. *Reproduction.* 133(2):331-59.

Witt,D.M. & Insel,T.R. (1994) Increased Fos Expression in Oxytocin Neurons Following Masculine Sexual-Behavior. *Journal of Neuroendocrinology*, 6, 13-18.

Wu,X.H. & Hu,L.Q. (2002) Effect of androgen on penile erection induced by L-arginine in paraventricular nucleus. *Zhonghua Nan.Ke.Xue.*, 8, 270-272.

Xiao,M., Ding,J., Wu,L., Han,Q., Wang,H., Zuo,G. & Hu,G. (2005) The distribution of neural nitric oxide synthase-positive cerebrospinal fluid-contacting neurons in the third ventricular wall of male rats and coexistence with vasopressin or oxytocin. *Brain Res.*, 1038, 150-162.

Xing,J.P., Cui,X.F., Sun,J.H., Li,Q., Liu,J. & Qiu,S.D. (2005) Intracavernous pressure responses induced by electrical stimulation of penile dorsal nerves and intracavernosal drug injection in rats. *Zhonghua Nan.Ke.Xue.*, 11, 281-3, 287.

Yanagimoto,M., Honda,K., Goto,Y. & Negoro,H. (1996) Afferents originating from the dorsal penile nerve excite oxytocin cells in the hypothalamic paraventricular nucleus of the rat. *Brain Research*, 733, 292-296.

Yang,C.R., Bourque,C.W. & Renaud,L.P. (1991) Dopamine D2 receptor activation depolarizes rat supraoptic neurones in hypothalamic explants. *J.Physiol*, 443, 405-419.

Yoshimura,R., Kiyama,H., Kimura,T., Araki,T., Maeno,H., Tanizawa,O. & Tohyama,M. (1993) Localization of oxytocin receptor messenger ribonucleic acid in the rat brain. *Endocrinology.* 133(3):1239-46.

Zhang,X.H., Filippi,S., Vignozzi,L., Morelli,A., Mancina, R., Luconi,M., Donati,S., Marini,M., Vannelli,G.B., Forti,G. & Maggi,M.(2005) Identification, localization and functional in vitro and in vivo activity of oxytocin receptor in the rat penis. *J Endocrinol.*184(3):567-76.